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| (54) Title: ANTIVIRAL RICIN-LIKE PROTEINS | | | |
| (57) Abstract | | | |
| <p>The present invention provides a protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence contains a cleavage recognition site for a retroviral protease such as HIV or an HTLV protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cells infected with a retrovirus utilizing the proteins of the invention; and pharmaceutical compositions for treating HIV infections and human T-cell leukemias involving HTLV.</p> | | | |
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- 1 -

Title: Antiviral Ricin-Like Proteins

FIELD OF THE INVENTION

The invention relates to proteins having A and B chains of a ricin-like toxin, linked by a linker sequence which is specifically cleavable by a retroviral protease to release the active A chain. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cells infected with a retrovirus utilizing the proteins of the invention and pharmaceutical compositions for treating HIV infection.

BACKGROUND OF THE INVENTION

Bacteria and plants are known to produce cytotoxic proteins which may consist of one, two or several polypeptides or subunits. Those proteins having a single subunit may be loosely classified as Type I proteins. Many of the cytotoxins which have evolved two subunit structures are referred to as type II proteins(Saelinger, C.B. in *Trafficking of Bacterial Toxins* (eds. Saelinger, C.B.) 1-13 (CRC Press Inc., Boca Raton, Florida, 1990). One subunit, the A chain, possesses the toxic activity whereas the second subunit, the B chain, binds cell surfaces and mediates entry of the toxin into a target cell. A subset of these toxins kill target cells by inhibiting protein biosynthesis. For example, bacterial toxins such as diphtheria toxin or *Pseudomonas* exotoxin inhibit protein synthesis by inactivating elongation factor 2. Plant toxins such as ricin work by directly inactivating ribosomes [Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.); 51-105 (Elsevier Biomedical Press, Amsterdam, 1982].

Ricin, derived from the seeds of *Ricinus communis* (castor oil plant), is the most potent of the plant toxins. It is estimated that a single ricin A chain is able to inactivate ribosomes at a rate of 1500 ribosomes/minute. Consequently, a single molecule of ricin is enough to kill a cell (Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.) 51-105 (Elsevier Biomedical Press, Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. Biol. Chem. 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al. Biol. Chem. 261:7912 (1986)).

Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preroricin) with a 35 amino acid N-terminal

- 2 -

presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M. *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M. *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies
5 where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is secreted from the plant cells. The A chain is inactive in the proricin (O'Hare, M., et al. *FEBS Lett.* 273:200-204
10 (1990)) and it is inactive in the disulfide-linked mature ricin(Richardson, P.T., et al. *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The exact mechanism of
15 A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described (Rutenber, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Bio.* 244:410-422, 1994; Lamb and Lord *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al. *Nucleic Acids Res.* 13:8019 (1985)). Due to its extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for destroying or inhibiting target cells or organisms (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell-binding component, such as a
25 monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule
30 (i.e. both chains) and with the ricin A chain alone (Spooner et al. *Mol. Immunol.* 31:117-125, (1994)).

Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of the B chains in binding to the cell surface and in
35 translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104(1987); Vitetta & Thorpe *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these conjugates also promotes the entry of the

immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain binds nonspecifically to N-glycosylated galactose, present on most cells. IT-As are more specific and safer to use than IT-Rs. However, in the absence of the B chain the A chain has greatly reduced toxicity.

5 A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells. A major problem with the use of ITs is that often the target antigen is also found on non-tumour cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, due to the reduced potency of IT-As as compared to ITRs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and 10 production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104(1987)). IT-As and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

15 The insertion of intramolecular cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic the way that the natural toxin is activated. European patent application no. 466,222 describes the use of maize-derived pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor Xa, thrombin or collagenase. Westby et al. (*Bioconjugate Chem.*, 3:375-381, 1992) documented fusion proteins which have a specific cell binding component and proricin with a protease sensitive cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (FEBS Lett. 273:200-204, 1990) also describe a recombinant fusion protein of RTA and staphylococcal protein A joined by a trypsin-sensitive cleavage site. In view of the prevalence of the extracellular proteases utilized in these approaches, such artificial activation of the toxin precursor or immunotoxin do not confer a mechanism 20 for intracellular toxin activation, and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.

25 In view of the extreme toxicity of proteins such as ricin, the lack of specificity of the immunotoxins may severely limit their usefulness as therapeutics for the treatment of cancer and infectious diseases. The preparation of a suitable specific cell binding component may be problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition.

30 The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly AIDS, has been investigated. Bacterial toxins such as *Pseudomonas* exotoxin-A and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant 35 toxins, such as ricin and single chain ribosomal inhibitory proteins such as trichosanthin and poke weed antiviral protein have been used for the elimination of HIV infected cells (Olson et al. 1991, *AIDS Res. and Human Retroviruses* 7:1025-1030). The high toxicity of

these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

5 Immunotoxins are designed such that their specificity of action is determined solely by the antibody component; antigen presenting cells are preferentially destroyed by the drug (Pastan et al., *Annals New York Academy of Sciences* 758:345-353 (1995)). The toxin protein of immunotoxin conjugates does not give the therapeutic any additional specificity of action; it will bring about the destruction of any cell it is delivered to.

SUMMARY OF THE INVENTION

10 The present inventors have prepared novel recombinant toxic proteins which are specifically toxic to cells infected with retroviruses and which do not depend for their specificity of action on a specific cell-binding component. The recombinant proteins of the invention have an A chain of a ricin-like toxin linked to a B chain by a linker sequence, which may be specifically cleaved by a retroviral protease within infected cells to activate the toxic A chain.

15 In one aspect, the present invention provides a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence is not a linker sequence of a ricin-like toxin, but rather the heterologous 20 linker sequence contains a cleavage recognition site for a retroviral protease. The A and or the B chain may be those of ricin.

25 In an embodiment, the cleavage recognition site is the cleavage recognition site for an HIV protease. In a particular embodiment, the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN. In further particular embodiments, the nucleic acid has the nucleotide sequence shown in Figure 8, Figure 9 or Figure 10.

30 In another embodiment, the cleavage recognition site is the cleavage recognition site for a human T-cell leukemia virus protease. In a particular embodiment, the linker amino acid sequence comprises SAPQVLPVMHPN or SKTKVLVVQPKN cleaved by a human T-cell leukemia virus-I (HTLV-I) protease; or, SKTKVLVVQPRN or STTQCFFPILHPN cleaved by a human T-cell leukemia virus-II (HTLV-II) protease.

35 The present invention further provides a plasmid incorporating the nucleic acid of the invention. In an embodiment, the plasmid has the restriction map as shown in Figure 1A, 2A, 3A, 16A, 17A, 18A, or 19A.

40 In another embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the invention. In particular embodiments, the invention provides a baculovirus transfer vector having the restriction map as shown in

- 5 -

Figures 5, 6, 7, 16C, 17C, 18C, or 19C or having the DNA sequence as shown in Figure 11.

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease. The A and/or the B chain may be those of ricin.

In another aspect, the invention provides a method of inhibiting or destroying mammalian cells infected with a retrovirus having a protease, comprising the steps of preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for the retrovirus protease and introducing the recombinant protein into the cells. In an embodiment, the retrovirus is HIV.

The present invention also relates to a method of treating a mammal infected with HIV by administering the recombinant proteins of the invention to the mammal.

Also provided is a process for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

In an embodiment, a process is provided for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of identifying a cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

In a further aspect, the invention provides a pharmaceutical composition for treating a retroviral infection, such as HIV, in a mammal comprising the recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

The invention also contemplates a method for treating cancer cells containing an HTLV protease comprising (a) preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for an HTLV

protease; an (b) introducing the recombinant protein into the cells. The method can be used to treat a mammal with human T-cell leukemias involving HTLV. Compositions for treating human T-cell leukemias involving HTLV comprising the recombinant protein of the invention having a heterologous linker sequence which contains a cleavage 5 recognition site for an HTLV protease, and a pharmaceutically acceptable carrier , diluent, or excipient are also provided.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred 10 embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:
15 Figure 1A summarizes the cloning strategy used to generate the pAP-146 construct;
Figure 1B shows the nucleotide sequence of the HIV-A linker region of pAP-146;
Figure 2A summarizes the cloning strategy used to generate the pAP-147 construct;
Figure 2B shows the nucleotide sequence of the HIV-B linker region of pAP-147;
Figure 3A summarizes the cloning strategy used to generate the pAP-148 construct;
20 Figure 3B shows the nucleotide sequence of the HIV-H linker region of pAP-148;
Figure 4 shows the amino acid sequences of the wild type ricin linker, the pAP-146 linker, the pAP-147 linker and the pAP-148 linker;
Figure 5 shows the subcloning of the HIV-A linker variant into a baculovirus transfer vector;
25 Figure 6 shows the subcloning of the HIV-B linker variant into a baculovirus transfer vector;
Figure 7 shows the subcloning of the HIV-H linker variant into a baculovirus transfer vector;
Figure 8 shows the DNA sequence of the pAP-190 insert;
30 Figure 9 shows the DNA sequence of the pAP-196 insert;
Figure 10 shows the DNA sequence of the pAP-197 insert;
Figure 11 shows the DNA sequence of the baculovirus transfer vector pVL1393;
Figure 12 is a diagram of the vector pSB2;
Figure 13 shows a Western Blot of a pAP-190 proricin variant;
35 Figure 14 is a blot showing cleavage of a pAp 190 proricin variant by HIV protease;
Figure 15 is a blot showing activation of pAP-190 proricin variant by HIV

protease;

Figure 16A is a diagram summarizing the cloning strategy used to generate the pAP-205 construct;

5 Figure 16B shows the nucleotide sequence of the HTLV-I-A linker regions of pAP-205;

Figure 16C is a diagram showing the subcloning of the HTLV-I-A linker variant into a baculovirus transfer vector;

10 Figure 16D shows the DNA sequence of the pAP-206 insert containing ricin and the HTLV-I-A linker;

Figure 17A is a diagram summarizing the cloning strategy used to generate the pAP-207 construct;

15 Figure 17B shows the nucleotide sequence of the HTLV-I-B linker regions of pAP-207;

Figure 17C is a diagram summarizing the subcloning of the HTLV-I-B linker variant into a baculovirus transfer vector;

Figure 17D shows the DNA sequence of the pAP-208 insert containing ricin and the HTLV-I-B linker;

20 Figure 18A is a diagram summarizing the cloning strategy used to generate the pAP-209 construct;

Figure 18B shows the nucleotide sequence of the HTLV-II-A linker regions of pAP-209;

25 Figure 18C is a diagram summarizing the subcloning of the HTLV-II-A linker variant into a baculovirus transfer vector;

Figure 18D shows the DNA sequence of the pAP-210 insert containing ricin and the HTLV-II-A linker;

Figure 19A is a diagram summarizing the cloning strategy used to generate the pAP-211 construct;

30 Figure 19B shows the nucleotide sequence of the HTLV-II-B linker regions of pAP-211;

Figure 19C is a diagram summarizing the subcloning of the HTLV-II-B linker variant into a baculovirus transfer vector;

Figure 19D shows the DNA sequence of the pAP-212 insert containing ricin and the HTLV-II-B linker; and

35 Figure 20 shows the amino acid sequences of the wild type ricin linker and HTLV protease-sensitive amino acid linkers contained in linkers pAP-205 to pAP-212.

DETAILED DESCRIPTION OF THE INVENTION

Nucleic Acid Molecules of the Invention

The present inventors have cloned and expressed novel nucleic acid molecules having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The heterologous linker sequence contains a cleavage recognition site for a retroviral protease such as a cleavage recognition site for HIV or a human T-cell leukemia virus protease.

The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The term "linker sequence" as used herein refers to an internal amino acid sequence within the protein encoded by the nucleic acid molecule of the invention which contains residues linking the A and B chain so as to render the A chain incapable of exerting its toxic effect, for example catalytically inhibiting translation of a eukaryotic ribosome. By heterologous is meant that the linker sequence is not a sequence native to the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker sequence is cleaved the A chain becomes active or toxic.

The nucleic acid molecule of the invention was cloned by subjecting a preproricin cDNA clone (pAP-144) to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., Eur. J. Biochem., 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame.

The preproricin cDNA was amplified using the upstream primer Ricin-99 (or Ricin-109 may be used) and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The use of the upstream primer Ricin-109 circumvents the subcloning step into vector pSB2. The purified PCR fragment encoding the preproricin cDNA was then ligated into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene).

The cloned PCR product containing the putative preproricin gene was confirmed by

DNA sequencing of the entire cDNA clone (pAP-144). The sequences and location of oligonucleotide primers used for sequencing are shown in Table 1.

The prororicin cDNA clone (pAP-144) was subjected to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type prororicin linker region was replaced with the three linker sequences, pAP-146, pAP-147 and pAP-148 shown in Figure 4. The linker regions of the variants encode an HIV protease cleavage recognition sequence (Slalka et al., *Cell*, 56:911-913, 1989). The mutagenesis and cloning strategy used to generate the linker variants pAP-146, pAP-147 and pAP-148 are summarized in Figures 1A and 1B, 2A and 10 2B and 3A and 3B respectively. The first step involved a DNA amplification using a set of mutagenic primers (HIVA 1, 2; HIVB 1, 2; HIVH 1, 2) in combination with the two flanking primers Ricin-99Eco and Ricin1729Xba. Restriction digested PCR fragments were gel purified and then ligated with PBluescript SK which had been digested with Eco RI and Xba I. Ligation reactions were used to transform competent XL1-Blue cells 15 (Stratagene). Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the mutant linker sequences were confirmed by DNA sequencing.

Recombinant clones were subcloned into vector pSB2. The three constructs obtained were pAP-151, pAP-159, and pAP-163, with each having the mutant linker found in pAP-146, pAP-147, and pAP-148 respectively.

20 The cloning strategy described above may also be applied to the preparation of recombinant clones containing a cleavage recognition site for a human T-cell leukemia virus protease. For example, recombinant clones pAP-205, pAP-207, pAP-209, and pAP-211 were prepared using a method similar to the one described above.

25 The nucleic acid molecule of the invention has sequences encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease. The nucleic acid may be expressed to provide a recombinant protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a retroviral protease, such as an 30 HIV protease or an HTLV protease.

35 The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains are published (Rutener, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Bio.* 244:410-422, 1994; Lamb and Lord *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al. *Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A and B chains of ricin-like proteins and analogs and homologs of A and B chains of ricin-like proteins and truncations thereof (i.e., ricin-like

- 10 -

proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

Another aspect of the invention provides a nucleotide sequence which hybridizes 5 under high stringency conditions to a nucleotide sequence encoding the A and/or B chains of a ricin-like protein. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may 10 be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. 15 Methods for cloning ricin-like toxins are known in the art and are described, for example, in E.P. 466,222. Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of A and B chains of ricin or ricin- 20 like toxins. It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. Suitable primers may be selected from the sequences encoding regions of ricin-like proteins which are highly conserved, as described for example in U.S. Patent No 5,101,025 and E.P. 466,222.

A nucleic acid can be amplified from cDNA or genomic DNA using these 25 oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). 30 cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL). It will be appreciated that the methods described above may be used to obtain the coding 35 sequence from plants, bacteria or fungi, preferably plants, which produce known ricin-like proteins and also to screen for the presence of genes encoding as yet unknown ricin-like proteins.

A sequence containing a cleavage recognition site for a retroviral protease may be

- 11 -

selected based on the retrovirus which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the retrovirus protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to 5 cleavage by a retroviral protease. An assay to identify peptides having cleavage recognition sites for HIV protease is described in PCT/US88/01849. The HIV protease encoded by the p17 gene of HIV and has the highly conserved Asp-Thr-Gly sequence characteristic of the active site of cellular aspartyl proteases. The HIV protease may be prepared by methods known in the art and used to test suspected cleavage recognition 10 sites. For example, a polypeptide containing the suspected cleavage recognition site may be incubated with the protease and the amount of cleavage product determined (DiLannit, 1990, J. Biol. Chem. 285: 17345-17354). Substrates for HIV proteases are described in U.S. Patent No. 5,235,039. The invention is not restricted to proteins including the cleavage 15 recognition site for HIV proteases, but includes recognition sites of other retroviral proteases, including proteases of members of the subfamilies oncovirinae, lentivirinae and spumavirinae for example from HTLV, AMV, RSV, BLV, FeLV and MMTV. Examples of retroviral proteases and conserved sequences thereof are provided, for example, in Katoh et al., (Nature 329:654-656).

A sequence containing a cleavage recognition site for an HTLV protease may be 20 selected using the conventional methods described herein. The preparation of human T-cell leukemia virus proteases, their substrates and enzymatic activity assay methodology have been described by Petit, S.C. et al. (J. Biol. Chem. 266:14539-14547 (1991)) and Blaha, I. et al. (FEBS Lett. 309:389-393 (1992)).

In an embodiment, the cleavage recognition site is the cleavage recognition site for 25 an HIV protease. In a particular embodiment, the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN. In further particular embodiments, the nucleic acid has the nucleotide sequence shown in Figure 8, Figure 9 or Figure 10.

In another embodiment, the cleavage recognition site is the cleavage recognition 30 site for a human T-cell leukemia virus protease. In a particular embodiment, the linker amino acid sequence comprises SAPQVLPVMHPN or SKTKVLVVQPKN cleaved by a human T-cell leukemia virus-I (HTLV-I) protease; or, SKTKVLVVQPRN or STTQCFFPILHPN cleaved by a human T-cell leukemia virus-II (HTLV-II) protease.

The nucleic acid molecule of the invention may be prepared by site directed 35 mutagenesis. For example, the cleavage site of a retroviral protease may be prepared by site directed mutagenesis of the homologous linker sequence of a proricin-like toxin. Procedures for cloning proricin-like genes, encoding a linker sequence are described in EP

466,222. Site directed mutagenesis may be accomplished by DNA amplification of mutagenic primers in combination with flanking primers. Suitable procedures using the mutagenic primers HIVA1, HIVB1 and HIVH1 are shown in Figures 1A to 3B, and Figures 16A, 16B, 17A, 17B, 18A, 19A and 19B.

5 The nucleic acid molecule of the invention may also encode a fusion protein. A sequence encoding a heterologous linker sequence containing a cleavage recognition site for a retroviral protease may be cloned from a cDNA or genomic library or chemically synthesized based on the known sequence of such cleavage sites. The heterologous linker sequence may then be fused in frame with the sequences encoding the A and B chains of the 10 ricin-like toxin for expression as a fusion protein. It will be appreciated that a nucleic acid molecule encoding a fusion protein may contain a sequence encoding an A chain and a B chain from the same ricin-like toxin or the encoded A and B chains may be from different toxins. For example, the A chain may be derived from ricin and the B chain may be derived from abrin. A protein may also be prepared by chemical conjugation of the A 15 and B chains and linker sequence using conventional coupling agents for covalent attachment.

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding an A and B chain and a linker into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein of the invention. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed in vitro with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

Recombinant Protein of the Invention

As previously mentioned, the invention provides novel recombinant proteins 25 which incorporate the A and B chains of a ricin-like toxin linked by a heterologous linker sequence containing a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease. It is an advantage of the recombinant proteins of the invention that they are non-toxic until the A chain is liberated from the B chain by specific cleavage of the linker by the retroviral protease, such as an HIV protease or an 30 HTLV protease. Thus the protein may be used to specifically target cells infected with the retrovirus in the absence of additional specific cell-binding components to target infected cells. It is a further advantage that the retroviral protease cleaves the heterologous linker intracellularly thereby releasing the toxic A chain directly into the 35 cytoplasm of the infected cell. As a result, infected cells are specifically targeted and non-infected cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein which blocks protein synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis*

- 13 -

(castor oil plant). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. *J. Biol. Chem.* 262:8128 (1987)). The 5 B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al. *Biol. Chem.* 261:7912 (1986)).

Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (proricin) with a 35 amino acid N-terminal 10 presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M. *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M. *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains 15 (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is secreted from the plant cells. The A chain is inactive in the proricin (O'Hare, M., et al. *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin(Richardson, P.T., et al. 20 *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include bacterial, fungal and plant toxins which have A and B 25 chains and inactivate ribosomes and inhibit protein synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is thought to facilitate entry of the A chain into the cell cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin are plant toxins which have one A 30 chain and one B chain. Examples of such toxins include abrin which may be isolated from the seeds of *Abrus precatorius*, ricin which may be isolated from the seeds of castor beans *Ricinus communis*, and modeccin.

Ricin-like bacterial proteins include diphtheria toxin, which is produced by 35 *Corynebacterium diphtheriae*, *Pseudomonas* enterotoxin A and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A chain. The recombinant proteins of the invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein

with the B chain of another toxin. Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, crotin II, curcin II and wheat germ inhibitor. Examples of fungal toxins having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents Nos. 5,101,025 and 5,128,460.

In addition to the entire B or A chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by proteolytic degradation, for example with Nagarse (Funmatsu et al., 1970, Jap. J. Med. Sci. Biol. 23:264-267). Similarly, the recombinant protein of the invention may contain only that portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in EP 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation. Non-glycosylated chains may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources,

including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be 5 readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, 10 and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with 15 a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the 20 concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for 25 expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion 30 moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of 35 the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E

binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus' *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β -lactamase (penicillinase) and lactose promoter system (see Chang et al., *Nature* 275:615, 1978), the trp promoter (Nichols and Yanofsky, *Meth in Enzymology* 101:155, 1983) and the tac promoter (Russell et al., *Gene* 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (see Bolivar et al., *Gene* 2:9S, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, *Meth in Enzymology* 101:20-77, 1983 and Vieira and Messing, *Gene* 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.). Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors

include pTrc (Amann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89).

Yeast and fungi host cells suitable for carrying out the present invention include,
5 but are not limited to *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and
various species of the genus *Aspergillus*. Examples of vectors for expression in yeast *S.*
cerevisiae include pYEpSec1 (Baldari. et al., (1987) Embo J. 6:229-234), pMFa (Kurjan and
Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al., (1987) Gene 54:113-123), and
10 pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast
and fungi are well known to those of ordinary skill in the art.(see Hinnen et al., PNAS
USA 75:1929, 1978; Itoh et al., J. Bacteriology 153:163, 1983, and Cullen et al.
(Bio/Technology 5:369, 1987)).

Mammalian cells suitable for carrying out the present invention include, among
others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO
15 (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells.
Suitable expression vectors for directing expression in mammalian cells generally include
a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2;
cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational
control sequences. Examples of mammalian expression vectors include pCDM8 (Seed, B.,
20 (1987) Nature 329:840) and pMT2PC (Kaufman et al. (1987), EMBOJ. 6:187-195).

Given the teachings provided herein, promoters, terminators, and methods for
introducing expression vectors of an appropriate type into plant, avian, and insect cells
may also be readily accomplished. For example, within one embodiment, the proteins of
the invention may be expressed from plant cells (see Sinkar et al., J. Biosci (Bangalore)
25 11:47-58, 1987, which reviews the use of *Agrobacterium rhizogenes* vectors; see also
Zambryski et al., Genetic Engineering, Principles and Methods, Hollaender and Setlow
(eds.), Vol. VI, pp. 253-278, Plenum Press, New York, 1984, which describes the use of
expression vectors for plant cells, including, among others, pAS2022, pAS2023, and
pAS2034).

30 Insect cells suitable for carrying out the present invention include cells and cell
lines from *Bombyx* or *Spodoptera* species. Baculovirus vectors available for expression of
proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983)
Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D.,
35 (1989) Virology 170:31-39). Some baculovirus-insect cell expression systems suitable for
expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human
transgenic animals such as, rats, rabbits, sheep and pigs (see Hammer et al. (Nature

315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866).

5 The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

10 The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease. Such a protein could be prepared other than by recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and purified from their 15 natural plant, fungal or bacterial source. Such A and B chains could be prepared having the glycosylation pattern of the native ricin-like toxin.

20 N-terminal or C-terminal fusion proteins comprising the protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the selected protein or marker protein as described herein. The recombinant protein of the invention may also be conjugated to other proteins by known techniques. For example the proteins may be coupled using heterobifunctional thiol-containing linkers as described in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-propionate) or N-25 succinimidyl-5-thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins such as immunoglobulins, hormones, growth factors, lectins, insulin, low density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Utility of the Nucleic Acid Molecules and Proteins of the Invention

30 The proteins of the invention may be used to specifically inhibit or destroy mammalian cancer cells or mammalian cells infected with a retrovirus. It is an advantage of the recombinant proteins of the invention that they have specificity for the infected cells without the need for a cell binding component. The ricin-like B chain of the recombinant proteins recognize galactose moieties on the cell surface and ensure that the 35 protein is taken up by the cell and released into the cytoplasm. When the protein is released into a non-infected cell, the A chain will remain inactive bound to the B chain. However, when the protein is released into a cell infected with a retrovirus or containing

an HTLV or HIV protease, the retroviral protease will cleave the cleavage recognition site in the linker, releasing the toxic A chain.

The specificity of a recombinant protein of the invention may be tested by treating the protein with the retroviral protease, such as HIV protease or HTLV protease which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. Retroviral proteases such as HIV protease or HTLV protease may be isolated from infected cells or may be prepared recombinantly, for example following the procedures in Darket et al. (1988, J. Biol. Chem. 254:2307-2312). The cleavage products may be identified for example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an in vitro translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template. Toxicity of the cleavage products may be determined using a ribosomal inactivation assay (Westby et al. 1992, Bioconjugate Chem. 3:377-382). The effect of the cleavage products on protein synthesis may be measured in standardized assays of in vitro translation utilizing partially defined cell free systems composed for example of a reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA template and amino acids. Use of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, FEBS Lett. 1990, 273:200-204).

The ability of the recombinant proteins of the invention to selectively inhibit or destroy mammalian cells infected with a retrovirus such as cancer cells associated with HTLV or cells associated with HIV may be readily tested in vitro using mammalian cell cultures infected with the retrovirus of interest, or cancer lines. The selective inhibitory effect of the recombinant proteins of the invention may be determined by demonstrating the selective inhibition of viral antigen expression in mammalian cells, or selective inhibition of cellular proliferation in cancer cells or infected cells. For example, a selective inhibitory effect may be demonstrated by the selective inhibition of viral antigen expression in HIV-infected mononuclear phagocytic lineage cells; selective inhibition of cellular proliferation as measured against protein and DNA synthesis levels in treated, noninfected T cells and; selective loss of T cell viability. For example, the below-noted culture systems may be used to test the ability of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for the HIV protease to selectively inhibit HIV infected cells. The term HIV refers to a CD4+ dependent human immunodeficiency retrovirus, such as HIV-1 and variants thereof.

Normal human T lymphocytes may be prepared from peripheral blood samples and cultured in vitro, as generally described in U.S. Patent No. 4,869,903. HIV infected

cells may also be obtained from AIDS patients. The cells may be infected in vitro with HIV derived from an AIDS patient. The toxicity of the recombinant protein for infected and non-infected cultures may then be compared. HIV-infected T cells express HIV envelope protein on the cell surface, in particular the proteins gp120 and gp41. The 5 ability of the recombinant protein of the invention to inhibit the expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Toxicity may be measured based upon cell death or lysis, or by a reduction in the expression of HIV antigens, such as the major envelope proteins gp120 and gp41 or the HIV core protein antigen p24.

10 Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903). Similar assays may be carried out using other suitable mammalian cells which can be cultured in vitro and which are capable of maintaining retroviral replication. Examples of suitable 15 cells include mononuclear phagocytic lineage cells. Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al. 1990, FEBS Lett. 273:200-204). Infected cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as 20 a measure of cellular proliferation.

In the models of viral infection and replication for confirming the activity of the recombinant proteins of the invention, suitable mammalian cells used as hosts are those cells which can be cultured in vitro and which are capable of maintaining viral replication. Examples of suitable cells can be human T lymphocytes or mononuclear 25 phagocytic lineage cells. Normal human T lymphocytes may be prepared from peripheral blood samples and cultured in vitro, as generally described in U.S. Patent No. 4,869,903. Virally infected cells may also be obtained from the blood of infected patients. The toxicity of the recombinant protein for infected and non-infected cultures may then be compared. The ability of the recombinant protein of the invention to inhibit the 30 expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903).

35 Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al. 1990, FEBS Lett. 273:200-204). Infected

cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as a measure of cellular proliferation. In addition, toxicity may be measured based on cell viability, for example the viability of infected and non-infected cell cultures exposed to the recombinant protein may be 5 compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

Although, the specificity of the proteins of the invention for retrovirally infected cells is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to 10 the proteins of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include antibodies to retroviral proteins, or to cancer cell proteins.

Antibodies having specificity for a cell surface protein may be prepared by 15 conventional methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored 20 by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be 25 harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 30 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)]. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The term "antibody" as used herein is intended to include fragments thereof which 35 also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as

described above. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with cell surface components. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature 348, 552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or fragments thereof.

The proteins of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are

outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired 5 result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the 10 exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of 15 enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for 20 example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

25 The pharmaceutical compositions may be used in methods for treating mammals, including humans, infected with a retrovirus. It is anticipated that the compositions will be particularly useful for treating patients infected with HIV-1, HIV-2 or cancers involving retroviruses, such as human T-cell leukemias involving HTLV. The efficacy of such treatments may be monitored by assessing the health of the patient treated and by 30 measuring the percentage of HIV positive monocytes in treated patients.

The dose of the recombinant protein to be administered will depend on a variety of 35 factors which may be readily monitored in human subjects. Such factors include HIV antigen levels associated with HIV infected T cells or mononuclear phagocytes; HIV antigen levels in the bloodstream; reverse transcriptase activity associated with HIV-infected T cells or mononuclear phagocytes; and the ratio of viable HIV infected cells to uninfected cells. HIV antigen levels in plasma, for example, may be readily determined using an ELISA assay.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

EXAMPLE 1

Cloning and Expression of Prorcin Variants Activated by HIV Proteases

5 **Isolation of total RNA**

The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Maniatis et al., *Molecular Cloning: A Lab Manual* (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA generated using reverse transcriptase.

10 **cDNA Synthesis:**

Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.*, 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis was primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA was used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

15 **DNA Amplification and Cloning**

The first strand cDNA synthesis reaction was used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was amplified using the upstream primer Ricin-99 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product was fractionated on an agarose gel (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones were confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA was extracted using a

Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing

The cloned PCR product containing the putative preproricin gene was confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing was performed using an
5 Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing were as follows: Ricin267, Ricin486, Ricin725, Ricin937, Ricin1151, Ricini1399, Ricin1627, T3 primer
(5'AATTAACCCTCACTAAAGGG-3') and T7 primer
10 (5'GTAATACGACTCACTATAGGGC-3'). Sequence data was compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is shown in Table 1.

Mutagenesis of Preproricin Linker

The preproricin cDNA clone (pAP-144) was subjected to site-directed mutagenesis
15 in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region was replaced with the three linker sequences, pAP-146, pAP-147 and pAP-148 displayed in Figure 4. The linker regions of the variants encode an HIV protease cleavage recognition sequence (Slalka et al., Cell, 56:911-913, 1989). The mutagenesis and cloning strategy used to generate the
20 linker variant pAP-146 is summarized in Figures 1A and 1B. The mutagenesis and cloning strategy used to generate the linker variant pAP-147 is summarized in Figures 2A and 2B. The mutagenesis and cloning strategy used to generate the linker variant pAP-148 is summarized in Figures 3A and 3B. The first step involved a DNA amplification using a set of mutagenic primers (HIVA1; HIVB1; HIVH1) in combination with the two flanking
25 primers Ricin-99Eco and Ricin1729Xba. The PCR protocol and conditions used were the same as described above. PCR products from each mutagenesis reaction were gel purified then restriction digested with either Eco R1 for the A-chain encoding fragment, or Xba I for the B chain encoding fragment. Restriction digested PCR fragments were gel purified and then ligated with PBluescript SK which had been digested with Eco RI and Xba I.
30 Ligation reactions were used to transform competent XL1-Blue cells (Stratagene). Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the mutant linker sequence were confirmed by DNA sequencing.

Subcloning Preproricin Mutants into Vector pSB2

Full length preproricin cDNA was created from clones pAP-146, pAP-147, and
35 pAP-148, which lack the first three nucleotides of the signal sequence (Halling et al, Nucleic Acids Research, 13:8019-8033, 1985). The missing ATG (start codon) was introduced into each mutant by site-directed mutagenesis using primers Ricin-109 and

Ricin1729C. The DNA template for each reaction was pAP-146, pAP-147, or pAP-148, and the PCR conditions were the same as described above. PCR products were gel purified and then ligated with Sma I-digested pSB2 (see Figure 12). Recombinant clones were identified by restriction digests of plasmid miniprep DNA, and the 5' and 3' junctions 5 confirmed by DNA sequencing. The three constructs obtained were pAP-151, pAP-159, and pAP-163, with each having the mutant linker found in pAP-146, pAP-147, and pAP-148 respectively.

Subcloning Preproricin Mutants into Vector pVL1393

Preproricin variants were subcloned into the baculovirus transfer vector pVL1393 10 (PharMingen, sequence shown in Figure 11). The subcloning strategy for the HIV-A linker variant is summarized in Figure 5. The subcloning strategy for the HIV-B linker variant is summarized in Figure 6. The subcloning strategy for the HIV-H linker variant is summarized in Figure 7. The 1315 bp Eco RI/Kpn I fragment encoding the ricin A-chain 15 and each mutant linker was isolated from each of the variant clones in pSB2 (pAP-151, pAP-159, and pAP-163). Each of these purified fragments was ligated with a 564 bp KpnI/PstI fragment obtained from pAP-144, and with Eco RI/Pst I cleaved pVL1393. Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the 5' and 3' junctions confirmed by DNA sequencing. The three constructs obtained were 20 pAP-190, pAP-196, and pAP-197, each having the mutant linker found in pAP-146, pAP-147, and pAP-148, respectively.

Isolation of Recombinant Baculoviruses

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI-TN-581-4 25 (High Five)) were maintained on TMN-FH medium supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA (pAP-190, pAP-196, or pAP-197) was co-transfected with 0.5 30 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2×10^6 Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a 35 multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and

Gruenwald et al., *Baculovirus Expression Vector System: Procedures and Methods Manual*, 2nd Edition, (San Diego, CA, 1993)).

Expression of Mutant Prorcin

Recombinant baculoviruses (pAP-190baculo, pAP-196baculo, and pAP-197-baculo) 5 were used to infect 2×10^5 Tn368 or sf9 cells of an moi of 5 in EX-CELL400 media (JRH Biosciences) with 25mM α -lactose in spinner flasks. Media supernatants containing mutant proricins were collected on day 6 post-infection.

Purification of Mutant Prorcin

Media supernatants were ultracentrifuged at 100,000g for 1 hour. After the 10 addition of 1 mM phenylmethylsulfonyl fluoride, the supernatants were concentrated using an Amicon 8050 Ultrafiltration Cell fitted with a Diaflo XM50 membrane. Supernatants were then dialysed extensively against 137 mM NaCl, 2.2 mM KCl, 2.6 mM KH₂PO₄, and 8.6 mM Na₂HPO₄, pH 7.4 containing 1 mM dithiothreitol (dialysis buffer). Recombinant prorcin proteins were purified by affinity chromatography using lactose 15 agarose (Sigma) as previously described for recombinant ricin-B chain (Ferrini et al., Eur. J. Biochem., 233:772-777, 1995). Fractions containing recombinant prorcin were identified using SDS/PAGE, (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989) and by Western blot analysis using anti-ricin antibodies (Sigma).

20 In Vitro HIV Protease Digestion of Prorcin Variants

Affinity -purified mutant prorcin was treated with HIV protease to confirm specific cleavage in the linker region. Prorcin variants were eluted from the lactose- 25 agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Prorcin substrate was then incubated at 37°C for 60 minutes with 400 ng/ml recombinant HIV protease (BACHEM Biosciences Inc.). The cleavage products of prorcin (ricin A and B chains) were identified using SDS/PAGE (Sambrook et al., *Molecular Cloning: a Laboratory Manual*, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

30 In Vitro Translation Assay

The activity of protease-treated prorcin variants was monitored using a rabbit reticulocyte lysate in a non-radioactive (Amersham, ECL system) *in vitro* translation assay. Protease-treated prorcin was added to a standard 50 μ l translation reaction mix containing Brome Mosaic Virus mRNA as template (following the manufacturer's protocol). Active ricin variants inhibit the *in vitro* translation reaction by inactivating 35 ribosomes. Therefore, in the presence of an active ricin variant, no viral proteins are synthesized.

EXAMPLE 2

Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post transfection. The cells were removed by centrifuging the media at 1465 g for ten minutes using a SLA-1500 (Sorvall) centrifuge rotor. The supernatant was further clarified by centrifuging at 7970g for fifteen minutes.

5 Protease inhibitor phenylmethyl-sulfonyl fluoride (Sigma) was added to a final concentration of 1%. The samples were concentrated (five-fold) and dialyzed (four times five-fold) into dialysis buffer (1X baculo buffer (8.6 mM Na₂HPO₄, 2.6 mM KH₂PO₄, 137 mM NaCl and 2.6 mM KCl, pH 7.4) containing 2.5 mM lactose, and 0.02% NaN₃) using a MINITAN concentrator (Millipore) with 30kDa NMWL plates. Dithiothreitol (DTT)

10 was then added to a final concentration of 1 mM, and the samples were centrifuged at 37000g for one hour.

Following centrifugation, dialysis buffer containing 1 mM DTT was added to the samples to a final volume of 500 mL. The samples were degassed and applied overnight at 4°C to an ASF-sepharose affinity column (prepared according to Pharmacia protocol) in 15 a 10 mL chromatography column (Biorad). The column was washed with 300 mL of wash buffer (100 mM NaOAc, pH 5.2, 1mM DTT, and 0.02% NaN₃). Elution of pro-ricin variant was performed by applying 500 mL of elution buffer (100 mM NaOAc, pH 5.2, 250 mM lactose, and 5 mM DTT). The eluate was concentrated using an Amicon 8050 concentrator (Amicon) with a YM10 176 mm membrane, utilizing argon gas to pressurize the chamber.

20 The samples were further concentrated and dialyzed into 1X Baculo buffer using Ultrafree-15 Biomax (Millipore) 10 kDa NMWL filter devices, which were spun in a Beckman S4180 rotor (Beckman) at 2000g. Samples were flash frozen in dry ice and stored at -20°C.

Purification of pAP 190 by gel filtration chromatography

25 In order to purify the pro-ricin variant from processed material produced during fermentation, the protein was applied to a SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 50 mM Tris, 100mM NaCl, pH 7.5 containing 100 mM Lactose and 0.1% β-mercaptoethanol (βME). The flow rate of the column was 0.15 mL/min and fractions were collected every 25 minutes. The UV 30 (280 nm) trace was used to determine the approximate location of the purified pAP 190 and thus determine the samples for Western analysis.

Western analysis of column fractions

35 Fractions eluted from the SUPERDEX columns (Pharmacia) were analyzed for purity using standard Western blotting techniques. An aliquot of 10μL from each fraction was boiled in 1X sample buffer (62.6 mM Tris-C1, pH 6.8, 4.4% βME, 2% sodium dodecyl sulfate (SDS), 5% glycerol (all from Sigma) and 0.002% bromophenol blue (Biorad)) for

five minutes. Denatured samples were loaded on 12% Tris-Glycine Gels (Biorad) along with 50 ng of RCA₆₀ (Sigma) and 5 µL of kaleidoscope prestained standards (Biorad). Electrophoresis was carried out for ninety minutes at 100V in 25 mM Tris-Cl, pH 8.3, 0.1% SDS, and 192 mM glycine using the BioRad Mini Protean II cells (Biorad).

5 Following electrophoresis gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% Methanol) for a few minutes. PVDF Biorad membrane was presoaked for one minute in 100% methanol and two minutes in transfer buffer. Whatman paper was soaked briefly in transfer buffer. Five pieces of Whatman paper, membrane, gel, and another five pieces of Whatman paper were arranged on the bottom 10 cathode (anode) of the Pharmacia Novablot transfer apparatus (Pharmacia). Transfer was for one hour at constant current (2 mA/cm²).

Transfer was confirmed by checking for the appearance of the prestained standards on the membrane. Non-specific sites on the membrane were blocked by incubating the blot for thirty minutes in 1X Phosphate Buffered Saline (1X PBS; 137 mM 15 NaCl, 2.7 mM KC1, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) with 5% skim milk powder (Carnation). Primary antibody (Rabbit α-ricin, Sigma) was diluted 1:3000 in 1X PBS containing 0.1% Tween 20 (Sigma) and 2.5% skim milk and incubated with blot for forty five minutes on a orbital shaker (VWR). Non-specifically bound primary antibody was removed by washing the blot for ten minutes with 1X PBS containing 0.2% Tween 20. This 20 was repeated four times. Secondary antibody donkey anti-rabbit (Amersham) was incubated with the blot under the same conditions as the primary antibody. Excess secondary antibody was washed as described above. Blots were developed with the ECL Western Blotting detection reagents according to the manufacturer's instructions. Blots were exposed to Medtec's Full Speed Blue Film (Medtee) or Amersham's ECL Hyperfilm 25 (Amersham) for three to fifteen minutes. Film was developed in a KODAK Automatic Developer.

Determination of lectin binding ability of pro-ricin variant

An Immulon 2 place (VDVR) was coated with 100 µl per well of 10µg/ml of asialofetuin and left overnight at 4°C. The plate was washed with 3X 300 µL per well 30 with ddH₂O using an automated plate washer (BioRad). The plate was blocked for one hour at 37°C by adding 300 µL per well of PBS containing 1% ovalbumin. The plate was washed again as above. Pro-ricin variant pAP 190 was added to the plate in various dilutions in 1X Baculo. A standard curve of RCA₆₀ (Sigma) from 1-10 ng was also included. The plate was incubated for 1 h at 37°C. The plate was washed as above. Anti-ricin 35 monoclonal antibody (Sigma) was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin and 0.1% tween-20, added at 100 µL per well and incubated for 1 h at 37°C. The plate was

- 30 -

washed as above. Donkey-anti rabbit polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100 μ L per well and incubated for 1 h at 37°C. The plate was given a final wash as described above. Substrate was added to plate at 100 μ L per well (1 mg/ml o-phenylenediamine (Sigma), 1 μ L/ml H₂O₂, 25 μ L of 5 stop solution (20% H₂SO₄) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

Determination of pAP 190 activity using the rabbit reticulocyte assay

Ricin sample were prepared for reduction.

- A) RCA₆₀ = 3,500 ng / μ L of RCA₆₀ + 997 μ L 1xEndo buffer (25mM Tris,
10 25mM KCl, 5mM MgCl₂, pH 7.6)
Reduction = 95 μ L of 10ng/ μ L + 5 μ L β -mercaptoethanol
B) Ricin variants
Reduction = 40 μ L variant + 2 μ L β -mercaptoethanol
The ricin standard and the variants were incubated for 30 minutes
15 at room temperature.

Ricin - Rabbit Reticulocyte lysate reaction

The required number of 0.5 mL tubes were labelled. (2 tubes for each sample, + and - animline). To each of the sample tubes 20 μ L of 1X endo buffer was added, and 30 μ L of buffer was added to the controls. To the sample tubes either 10 μ L of 10ng/ μ L Ricin or 20 10 μ L of variant was added. Finally, 30 μ L of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the eppendorf tube and contents added into a 1.5 mL tube containing 1 mL of TRIZOL (Gibco). Samples were incubated for 15 minutes at room 25 temperature. After the incubation, 200 μ L of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 μ L of isopropanol. Samples were incubated for 15 minutes at room temperature and then centrifuged at 12,000 30 for 15 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C precipitated the RNA. All but approximately 20 μ L of the supernatant was removed and the remaining liquid evaporated using the speed vacuum machine. The control samples (-aniline) were dissolved in 10 μ L of 0.1 X E buffer (36 mM Tris, 30 mM NaH₂PO₄, 1 mM EDTA, pH 7.8) and stored at -70°C or on dry ice until later. Pellets from the other samples (+aniline samples) were dissolved in 20 μ L of DEPC treated ddH₂O. An 80 μ L aliquot of 1 M aniline 35 (distilled) with 2.8 M acetic acid was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA

was precipitated by adding 100 μ L of 95% ethanol and 5 μ L of 3M sodium acetate, pH 5.2 to each tube and centrifuging at 12,000 g for 30 minutes at 4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and excess liquid evaporated using the speed vacuum machine. These pellets (+ aniline samples) were dissolved in 10 μ L of 0.1 X E buffer. To all samples (+ and - aniline), 10 μ L of formamide loading dye was added. The RNA ladder (8 μ L of ladder + 8 μ L of loading dye) was also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer + 0.2% SDS. The gel was run for 10 90 minutes at 75 watts. RNA was visualized by staining the gel in 1 μ g/ μ L ethidium bromide in running buffer for 45 minutes. The gel was examined on a 302 nm UV box and photographed using the gel documentation system.

15 **Results:**
Protein Expression Yields

Aliquots were taken at each stop of the harvesting/purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results on an 800 mL prep of infected *T. ni* cells are given below.

| | <u>Aliquot</u> | <u>μg pAP 190</u> |
|----|-----------------------------------|----------------------------------|
| 20 | Before concentration and dialysis | 648.5 |
| | After concentration and dialysis | 364.4 |
| | ASF column flow through | 62.1 |
| | ASF column elution | 300.7 |

25 Yield: 300.7/648.5 = 46.4%

Purification of pAP 190 and Western Analysis of column fractions

Partially purified pAP 190 was applied to Superdex 75 and 200 (16/60) columns connected in series in order to remove the contaminating non-specifically processed pAP 190. Eluted fractions were tested via Western analysis and the fractions containing the most pure protein were pooled, concentrated and re-applied to the column. The variant was applied a total of three times to the column. The final purified pAP 190 has less than 1% processed variant. Figure 13 shows that the purified pAP 190 is in three fractions and the processed material eluted in two separate fractions.

30 The purified pAP 190 was tested for susceptibility to cleavage by HIV protease and for activation of the A-chain of the pro-ricin variant, (inhibition of protein

synthesis). PAP 190 was incubated with and without HIV protease for a specified time period and then electrophoresed and blotted. Cleaved pAP 190 will run as two 30 kDa proteins (B is slightly larger) under reducing (SDS-PAGE) conditions. Unprocessed pAP 190, which contains the linker region, will run at 60 kDa. HIV protease was able to 5 cleave the pAP 190 (shown in Figure 14). Lanes B and D show untreated; while lanes C and E to G show HIV protease treated pAP 190.

Activation of pAP 190 variant with HIV protease

Activation of HIV protease treated pAP 190 190, based on the method of *May et al.* (EMBO Journal. 8 301-8, 1989) was demonstrated in Figure 15. The appearance of the 10 390 based pair product is observed in lane B, which is the positive control, and not observed in lane C, the negative control. Lanes D-G show that there was no N-glycosidase activity in the pAP 190 variant as predicted. Lanes H-K show that processed pAP 190 possesses N-glycosidase activity as predicted.

The pAP 190 variant has been expressed in insect cells, purified to greater than 15 99%, and activation of the variant has been demonstrated by cleavage with HIV protease.

Example 3

Cloning and Expression of Prorcin Variants Activated by HTLV

Isolation of total RNA:

20 The preproricin gene is cloned from new foliage of the castor bean plant. Total messenger RNA is isolated according to established procedures (Maniatis et al., Molecular Cloning: A Lab Manual (Cold Spring Harbour Press, Cold Spring Harbour, 1989)) and cDNA generated using reverse transcriptase.

cDNA Synthesis:

25 Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides are synthesized using an Applied Biosystems Model 392 30 DNA/RNA Synthesizer. First strand cDNA synthesis is primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA is used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

DNA Amplification and Cloning:

35 The first strand cDNA synthesis reaction is used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA is amplified using the upstream primer Ricin-109 and the downstream primer Ricin1729C

with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification is carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product is fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA is then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RI-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones are confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA is extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing:

The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing is performed using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing are as follows: Ricin267, Ricin486, Ricin725, Ricin937, Ricin1151, Ricin 1399, Ricin1627, T3 primer (5' A A T T A A C C C T C A C T A A ^ G G G - 3') and T7 primer (5'GTAATACGACTCACTATAGGGC-3'). Sequence data is compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is shown in Table 1.

Mutagenesis of Preproricin Linker:

The preproricin cDNA clone (pAP-144) is subjected to site directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region is replaced with the linker sequences displayed in Figure 20. The linker regions of the variants encode a disease-specific protease cleavage recognition sequence (Slalka et al., *Cell*, 56:911-913, 1989). The mutagenesis and cloning strategy used to generate the HTLV protease-sensitive linker variants is summarized in Figures 16A, 17A, 18A and 19A.

The first step involves a DNA amplification using a set of mutagenic primers encoding for the disease-specific protease-sensitive linker in combination with the two flanking primers Ricin-109Eco and Ricin1729Xba. The PCR protocol and conditions used

are the same as described above. PCR products from each mutagenesis reaction are gel purified then restriction digested with either Eco R1 for the A-chain encoding fragment, or Xba I for the B chain encoding fragment. Restriction digested PCR fragments are gel purified and then ligated with pBluescript SK which has been digested with Eco RI and 5 Xba I. Ligation reactions are used to transform competent XL1-Blue cells (Stratagene). Recombinant clones are identified by restriction digests of plasmid miniprep DNA and the mutant linker sequence are confirmed by DNA sequencing.

Subcloning Preproricin Mutants into Vector pVL1393:

Preproricin variants are subcloned into the baculovirus transfer vector pVL1393 10 (PharMingen). The subcloning strategy for the HTLV protease-sensitive linker variants is summarized in Figures 16C, 17C, 18C, and 19C. The 1315 bp Eco RI/Kpn I fragment encoding the ricin A-chain and each mutant linker is isolated from pAP-205, pAP-207, pAP-209 or pAP-211. Each of these purified fragments is ligated with a 564 bp KpnI/PstI fragment obtained from pAP-144, and with Eco RI/Pst I cleaved pVL1393. Recombinant 15 clones are identified by restriction digests of plasmid miniprep DNA and the 5' and 3' junctions confirmed by DNA sequencing.

Isolation of Recombinant Baculoviruses:

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI TN-581-4 20 (High Five)) are maintained on TMN-FH medium supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA (pAP-190, pAP-196, or pAP-197) is co-transfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2 x 10⁶ Tn368 insect cells 25 following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media are centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants are then amplified by infecting Tn368 cells at a 30 multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants. A total of three rounds of amplification are performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 35 2nd Edition, (San Diego, CA, 1993)).

Expression of Mutant Proricin:

Recombinant baculoviruses (pAP-206-baculo, pAP-208-baculo, pAP-210-baculo, and pAP-212-baculo) are used to infect 2 x 10⁵ Tn368 or sf9 cells of an moi of 5 in EX-CELL400 media (JRH Biosciences) with 25mM α -lactose in spinner flasks. Media
5 supernatants containing mutant proricins are collected on day 6 post-infection.

Purification of Mutant Proricin:

Media supernatants are ultracentrifuged at 100,000g for 1 hour. After the addition of 1 mM phenylmethylsulfonyl fluoride, the supernatants are concentrated using an Amicon 8050 Ultrafiltration Cell fitted with a Diaflo XM50 membrane. Supernatants are
10 then dialysed extensively against 137 mM NaCl, 2.2 mM KCl, 2.6 mM KH₂PO₄, and 8.6 mM Na₂HPO₄, pH 7.4 containing 1 mM dithiothreitol (dialysis buffer). Recombinant proricin proteins are purified by affinity chromatography using lactose agarose (Sigma) as previously described for recombinant ricin-B chain (Ferrini et al., *Eur. J. Biochem.* 233:772-777 (1995)). Fractions containing recombinant proricin are identified using
15 SDS/PAGE, (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989) and by Western blot analysis using anti-ricin antibodies (Sigma).

In Vitro Protease Digestion of Proricin Variants:

Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. Ricin-like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate is then incubated at 37°C for 60 minutes with a disease-specific protease. The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook et al.,
25 Molecular Cloning: a Laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

HTLV proteases may be obtained from Bachem Bioscience. Cathepsin B may be obtained from Medcor or Calbiochem.

In Vitro Translation Assay:

The activity of protease-treated ricin-like toxin variants is monitored using a rabbit reticulocyte lysate in a non-radioactive (Amersham, ECL system) *in vitro* translation assay. Protease-treated proricin is added to a standard 50 ml translation reaction mix containing Brome Mosaic Virus mRNA as template (following the manufacturer's protocol). Active ricin variants inhibit the *in vitro* translation reaction by
30 inactivating ribosomes. Therefore, in the presence of an active ricin variant, no viral proteins are synthesized.
35

In Vitro Yeast Protein Synthesis Assay

The activity of protease-treated prorcin-like toxins may also be assessed by a yeast protein synthesis assay. For example, Murakami, S et al., Mol., Cel. Biol. 2:588-592, 1982, teaches a yeast protein synthesis assay to determine ricin-like toxicity which is as sensitive as mammalian cell assays.

5 Six five mL cultures of *Saccharomyces cerevisiae* (Y235 cells and 2 cell wall mutants) in YPD medium (10 g/L yeast extract, 20 g/L peptone) are started by inoculating 800 uL of medium with 1 colony of *Saccharomyces cerevisiae*, vortexing, then adding 100 uL of this suspension to 5 mL of medium. Cultures are grown overnight at 30°C with gentle agitation. Cells are expanded by inoculating 100 uL of YPD medium with one or more of
10 the 5 mL overnight cultures and are grown at 30°C with gentle agitation until a concentration of 1×10^5 cells/mL. Cells are washed with sterile double-distilled water, centrifuged at 1,200 g for 3 minutes and concentrated 3-fold in ZSM buffer(1 M sorbitol, 10 mM Tris-Cl, pH 7.5, 50 mM dithiotheitol (DDT)). Samples are incubated with gentle shaking for 10 minutes at 30°C, centrifuged at 1,200 g for 3 minutes and resuspended in ZSM
15 buffer such that the cell concentration was 1×10^8 cells/ml. Cell walls are disrupted by adding 1 mL of beta-glucuronidase (Sigma, St. Louis, MO) to the samples and incubating for 1 hour at room temperature with gentle agitation. Cells are washed 3 times with ZSM and protoplast cells resuspended in regeneration medium (0.17% yeast nitrogen base without amino acids (Difco, Detroit, Michigan), 2 Dropout + all (essential amino acids),
20 10 mM Tris-Cl, pH 7.5, 2% glucose, 1M sorbitol) to a final concentration of 1×10^8 cells/mL. An activated prorcin variant which has been dialysed in sterile 1X baculo buffer (0.137 M) NaCl, 2.7 mM KCl, 2.6 mM KH₂PO₄ pH 7.4) is added to one half of the protoplast, while sterile 1 X baculo buffer alone is added to the other half of the protoplasts as control. Both sets of samples are incubated at room temperature with gentle agitation. At
25 time periods of 0, 1, 2, and 3 hours, an aliquot of each culture is removed. The cells are diluted serially from 10^{-4} to 10^{-8} in ZSM and plated on soft agar (1:1 ZSM:YPD, 15% agar). Simultaneously, dilutions are made from 10^{-2} to 10^{-4} in sterile double-distilled water and 50 uL aliquots are plated onto YPD medium with 20% agar. Plates are incubated for 2 days at 30°C after which times colonies were counted. A plot of cell count vs. time is used to compare the ricin test culture vs. the control culture with no ricin.
30

The activated prorcin-like toxin variant inhibits *in vitro* protein synthesis through ribosomal inactivation. The rate of cell growth of the treatment group is expected to be substantially lower than that of the control group.

N-Glycosidase Activity of Prorcin Variants on rRNA Oligonucleotides

35 Ricin-like toxins inhibit ribosomal function by hydrolysing the N-glycosidic bond between the nucleotide base and the ribose at position A4319 in eukaryotic 28S ribosomal

RNA (rRNA). The ability of the activated ricin-like toxins to inhibit ribosomal RNA (rRNA) function may be examined in an *in vitro* ribonucleotide catalysis assay using a synthetic oligoribonucleotide possessing the secondary structure of the natural RNA hydrolytic cleavage domain.

5 A synthetic 32-nucleotide RNA oligomer (University of Calgary, DNA Core Services) that mimics the 28S rRNA toxin active site is used to test the N-glycosidase activity of proricin variants. The sequence of oligonucleotide and the general methodology are substantially as described in Gluck, A. and Wool I.G., *J. Mol. Biol.* 256:838-848, 1996.

10 A labelling reaction is set up to include: 50 pmol of oligonucleotide, 20 units of T4 polynucleotide kinase (PNK; Gibco-BRL, Gaithersburg, MA), 25 pmol of γ -³²P (Amersham, Arlington, IL), 1X T4 PNK buffer in a final volume of 50 uL. The samples are incubated for 30 minutes at 37°C and then for 20 minutes at 65°C. The labelled oligonucleotide is precipitated with 95% ethanol and dried using a thermal cycler. A 15 second ethanol precipitation step can be repeated to remove further trace contaminants. The RNA was resuspended to a final concentration of 1 ng/uL in 10 mM Tris-Cl (pH 7.6) and 50 mM NaCl (5 ng of oligonucleotide is used per sample).

15 Activated proricin variant is reduced in 1 X baculo buffer with 1% beta-mercaptoethanol for 30 minutes at room temperature prior to use. The oligonucleotides are heated at 90°C for 1 minute in 10 mM Tris-Cl (pH 7.6), 50 mM NaCl and allowed to renature at 0°C. CaCl₂, EGTA and water are added to the renatured RNA to give the following concentrations: 3 mM Tris-HCl (pH 7.6), 15 mM NaCl, 5 mM CaCl₂, and 5 mM EGTA. An activated proricin variant or ricin A-chain (Sigma, St. Louis, MO) is added to each tube. The concentration of the ricin ranged from 1-10 uM and the proricin variant 10-fold greater. The tubes are incubated at 35°C for 20 minutes and the reaction is stopped by the addition of sodium dodecylsulfate (SDS) at a final concentration of 0.5% (w/v). The oligonucleotide and 15 ug of added carrier tRNA (yeast tRNA; Gibco-BRL Gaithersburg, MA) are precipitated with 300 mM NaCl and 2.5 volumes of 95% ethanol. The pellets are washed once with 70% ethanol and dried on a CENTRIVAP (Labconco, Kansas City, MO). 20 The RNA is dissolved in 5 uL of water, 25 uL of a solution of aniline and acetic acid (1 and 2.8 mM respectively) is added and the sample is incubated for 10 minutes at 40°C. The aniline-treated RNA is precipitate with ethanol and 300 mM NaCl, washed once in 70% ethanol and dried on the CENTRIVAP. The pellets are dissolved in 10 uL of DEPC-treated double-distilled water and 10 uL of 2X loading dy (178 mM Tris-HCl (pH 8.3), 178 mM boric acid, 5 mM EDTA, 0.05% (w/v) bromophenol blue and 14 M urea), and are electrophoresed for 3 hours at 50 watts in 10% (w/v) polyacrylamide gel containing 7 M 25

urea in 1 X TBE buffer (89 mM Tris-HCl (pH 8.3), 89 mM boric acid, 2.5 mM boric acid, 2.5 mM EDTA). Gels are exposed to KODAK full speed blue X-ray film and left at -70°C. After 2 days, film was developed in a KODAK automatic film processor.

When proricin variant activated with a disease-specific protease is added to the oligoribonucleotide, hydrolysis of the N-glycosidic bond at position 20 (depurination of adenosine) would occur and appearance of two bands on the autoradiograph is expected. Proricin variant without pretreatment with the disease-specific protease would not cleave the RNA oligonucleotide and would result in a single band on the autoradiograph.

In Vitro Cytotoxicity Assay:

Human ovarian cancer cells (e.g. MA148) are seeded in 96-well flat-bottom plates and are exposed to ricin-like toxin variants or control medium at 37°C for 16 h. The viability of the cancer cells is determined by measuring [³⁵S]methionine incorporation and is significantly lower in wells treated with the toxin variants than those with control medium.

In Vivo Tumour Growth Inhibition Assay:

Human breast cancer (e.g. MCF-7) cells are maintained in suitable medium containing 10% fetal calf serum. The cells are grown, harvested and subsequently injected subcutaneously into ovariectomized athymic nude mice. Tumour size is determined at intervals by measuring two right-angle measurements using calipers.

In Vivo Tumour Metastasis Assay:

The metastasis study is performed substantially as described in Honn, K.V. et al. (*Biochem. Pharmacol.* 34:235-241 (1985)). Viable B16a melanoma tumour cells are prepared and injected subcutaneously into the left axillary region of syngeneic mice. The extent of tumour metastasis is measured after 4 weeks. The lungs are removed from the animals and are fixed in Bouin's solution and macroscopic pulmonary metastases are counted using a dissecting microscope. In general without therapeutic intervention, injection of 10⁵ viable tumour cells forms approximately 40-50 pulmonary metastases.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

- 39 -

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

- 40 -

TABLE 1

Table 1 - Sequence and Location of Oligonucleotide Primers

| Name of Primer | Primer Sequence [†] | Corresponds to proricin nucleotide numbers (see Figures 8-10) |
|-------------------|---|---|
| Ricin-109 | 5'-GGAGATGAAACCGGGAGGAAACTATTGTAAT-3' | 27 to 59 |
| Ricin-99Eco | 5' - <u>GCGGAATT</u> CCGGGAGGAAACTATTGTAAT - 3' | 37 to 59 |
| Ricin 267 | 5' - ACGGTTTATTTAGTTGA - 3' | 300 to 317 |
| Ricin486 | 5' - ACTTGCTGGTAATCTGAG - 3' | 519 to 536 |
| Ricin 725 | 5' - AGAATAGTTGGGGAGAC - 3' | 758 to 775 |
| Ricin937 | 5' - AATGCTGATGTTGTATG - 3' | 970 to 987 |
| Ricin1151 | 5' - CGGGAGTCTATGTGATGA - 3' | 1184 to 1201 |
| Ricin1399 | 5' - GCAAATAGTGGACAAGTA - 3' | 1432 to 1449 |
| Ricin1627 | 5' - GGATTGGTGTAGATGTG - 3' | 1660 to 1677 |
| Ricin1729C | 5' - ATAACTTGCTGTCCTTC - 3' | 1864 to 1846 |
| Ricin1729C Xba | 5' - <u>CGCTCTAGATAACTTGCTGTCCTTC</u> - 3' | 1864 to 1846 |

[†] underlined sequences inserted for subcloning purposes and not included in final proricin sequences

WE CLAIM:

1. A purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease.
5
2. The nucleic acid of claim 1 wherein the A chain is ricin A chain.
3. The nucleic acid of claim 1 wherein the B chain is ricin B chain.
4. The nucleic acid of claim 1 wherein the cleavage recognition site is the cleavage recognition site for an HIV protease.
- 10 5. The nucleic acid of claim 1 wherein the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN.
6. The nucleic acid of claim 1 having the nucleotide sequence shown in Figure 8, Figure 9 or Figure 10.
- 15 7. The nucleic acid of claim 1 wherein the cleavage recognition site is the cleavage recognition site for a human T-cell leukemia virus (HTLV) protease.
8. The nucleic acid of claim 7 wherein the linker amino acid sequence comprises SAPQVLPVMHPN; SKTKVLVVQPKN; SKTKVLVVQPRN or STTQCFFPILHPN.
9. A plasmid incorporating the nucleic acid of claim 1.
10. A plasmid as claimed in claim 7 having the restriction map as shown in Figure 20 1A, 2A, 3A, 16A, 17A or 18A.
11. A baculovirus transfer vector incorporating the nucleic acid of claim 1.
12. A baculovirus transfer vector as claimed in claim 11 having the restriction map as shown in Figure 5, 6, 7, 16C, 17C, or 18C.
13. A baculovirus transfer vector as claimed in claim 11 having the DNA sequence 25 as shown in Figure 11.

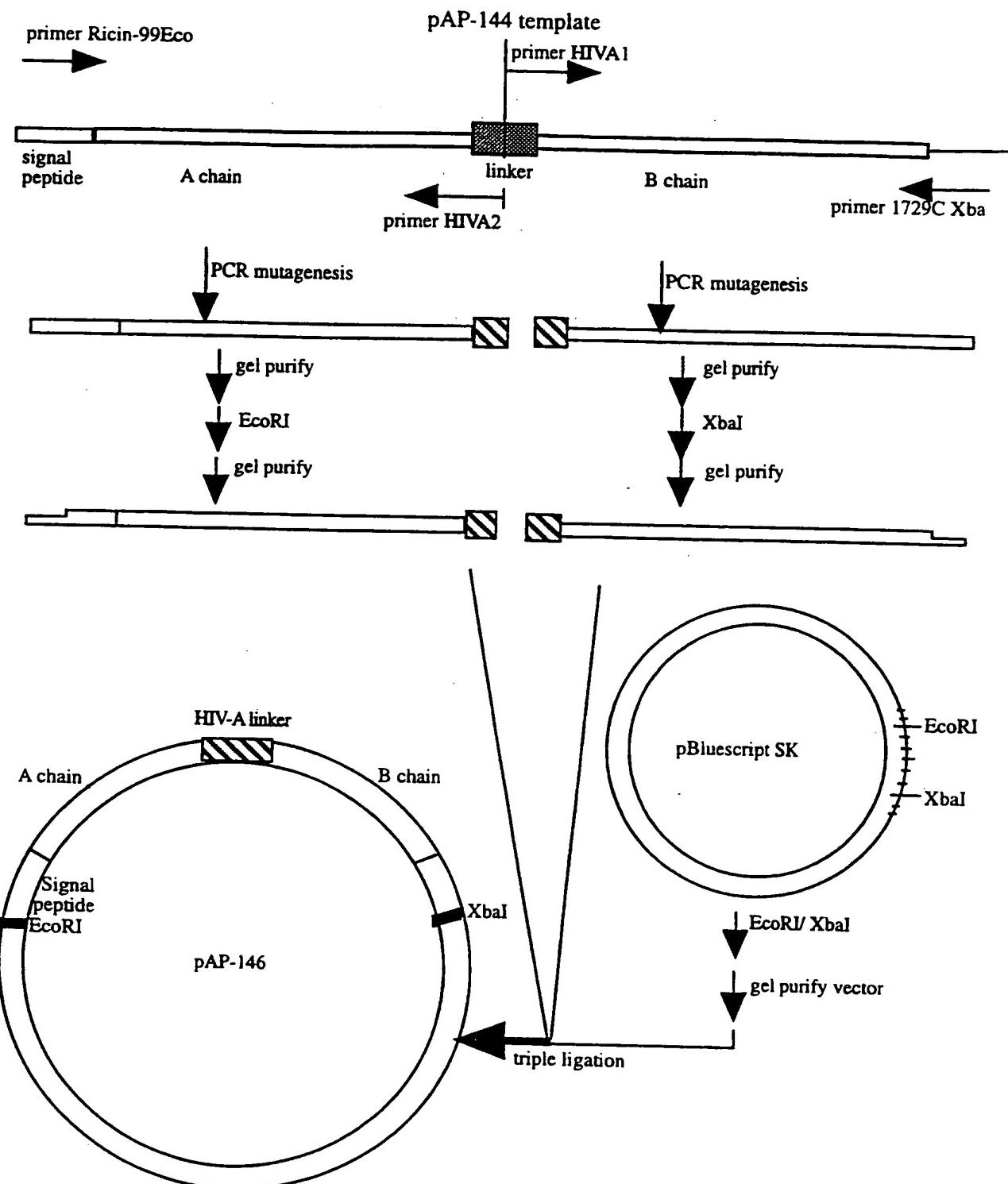
14. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease.
- 5 15. The recombinant protein of claim 14 wherein the A chain is ricin A chain.
16. The recombinant protein of claim 14 wherein the B chain is ricin B chain.
17. The recombinant protein of claim 14 wherein the cleavage recognition site is the cleavage recognition site for an HIV protease.
- 10 18. The recombinant protein of claim 14 wherein the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN.
19. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin- like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a HTLV protease
- 15 20. The recombinant protein of claim 19 wherein the linker amino acid sequence comprises SAPQVLPVMHPN; SKTKVLVVQPKN; SKTKVLVVQPRN or STTQCFPILHPN.
21. A method of inhibiting or destroying mammalian cells infected with a retrovirus having a protease, comprising the steps of preparing a recombinant protein of claim 14 wherein the linker sequence contains a cleavage recognition site for the retrovirus protease and administering the fusion protein to the cells.
- 20 22. A method as claimed in claim 21, wherein the retrovirus is HIV.
23. A method as claimed in claim 21 wherein the mammalian cells are human cells.
- 25 24. A method of treating a mammal infected with HIV, comprising the steps of preparing a recombinant protein of claim 14 and administering the protein to the

mammal.

25. A process for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease, and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.
- 10
26. A process for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of identifying a cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.
- 15
27. A pharmaceutical composition for treating a retroviral infection in a mammal comprising the recombinant protein of claim 14 and a pharmaceutically acceptable carrier, diluent or excipient.
- 20
28. A pharmaceutical composition for treating HIV infection in a mammal comprising the recombinant protein of claim 14 and a pharmaceutically acceptable carrier, diluent or excipient.

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1/47

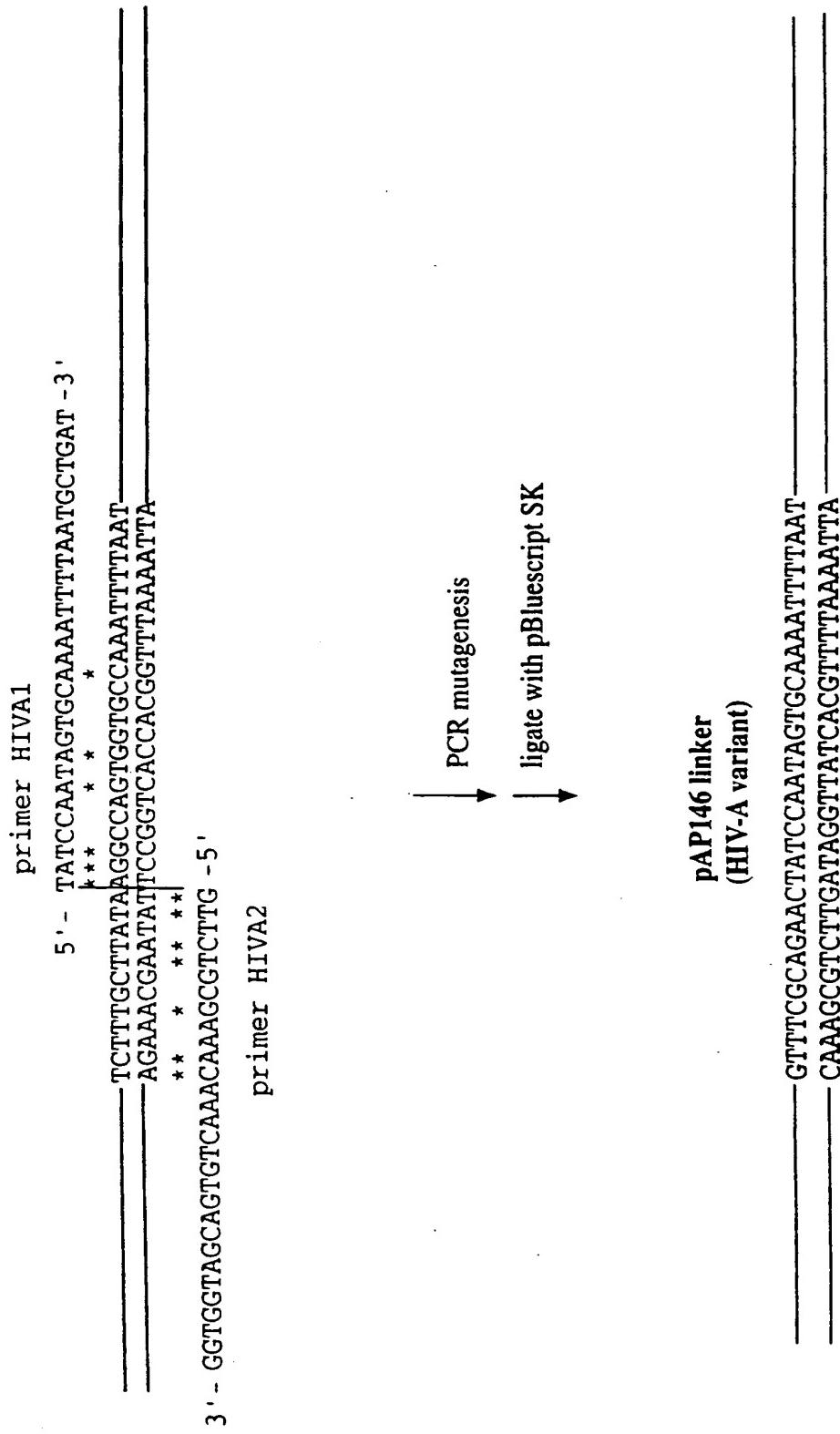
FIGURE 1A

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2/47

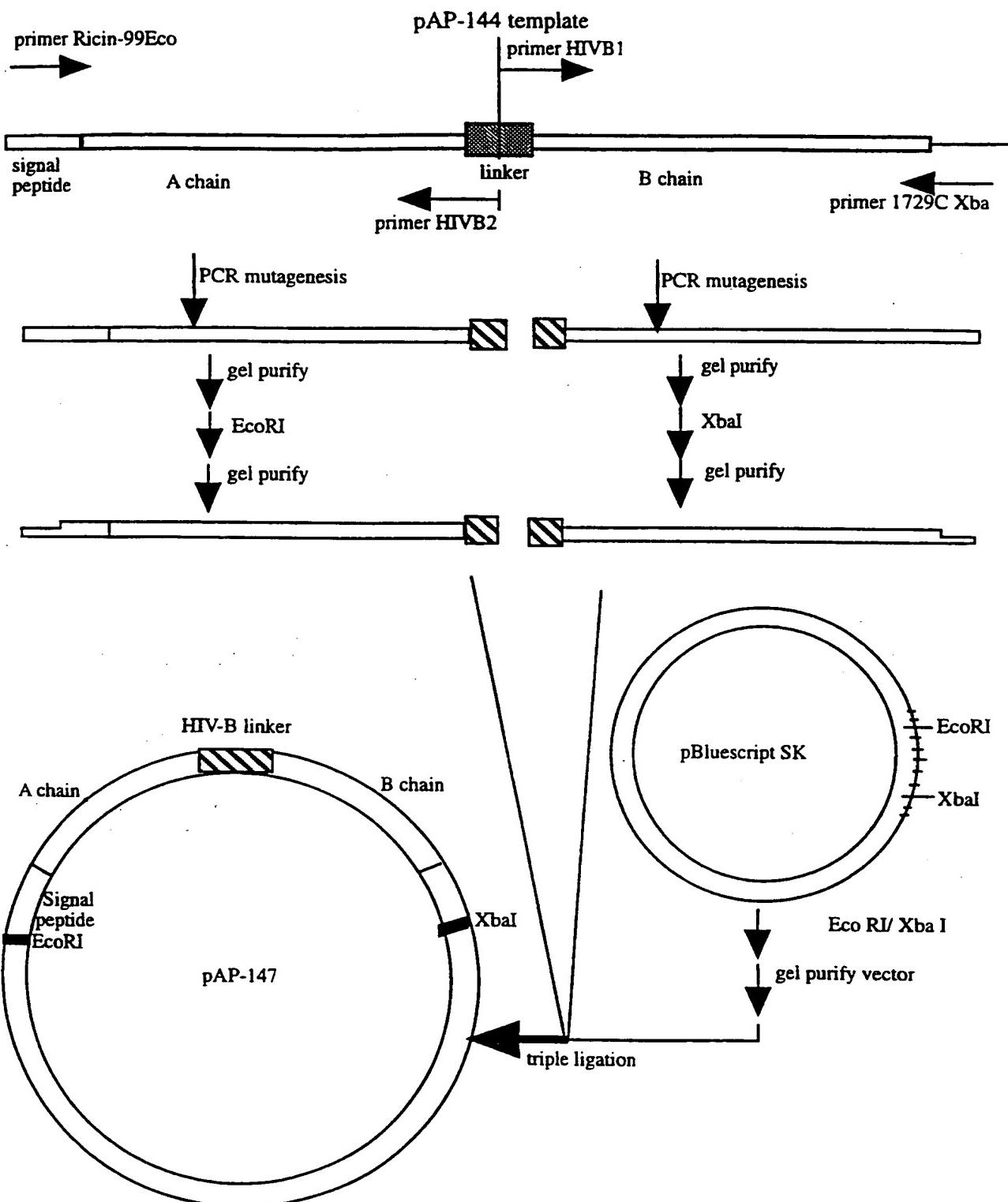
FIGURE 1B

WT preprotein linker



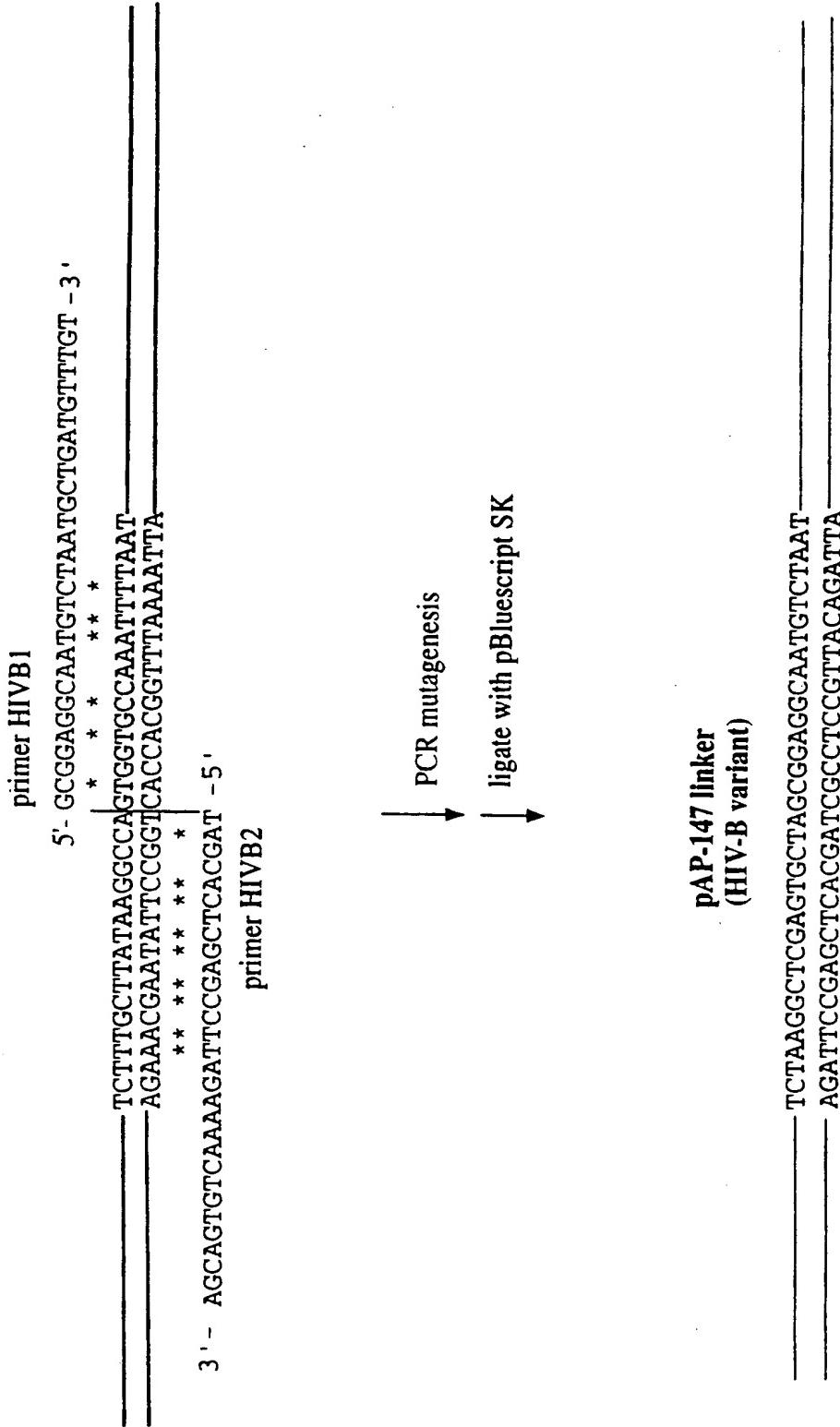
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3/47

FIGURE 2A

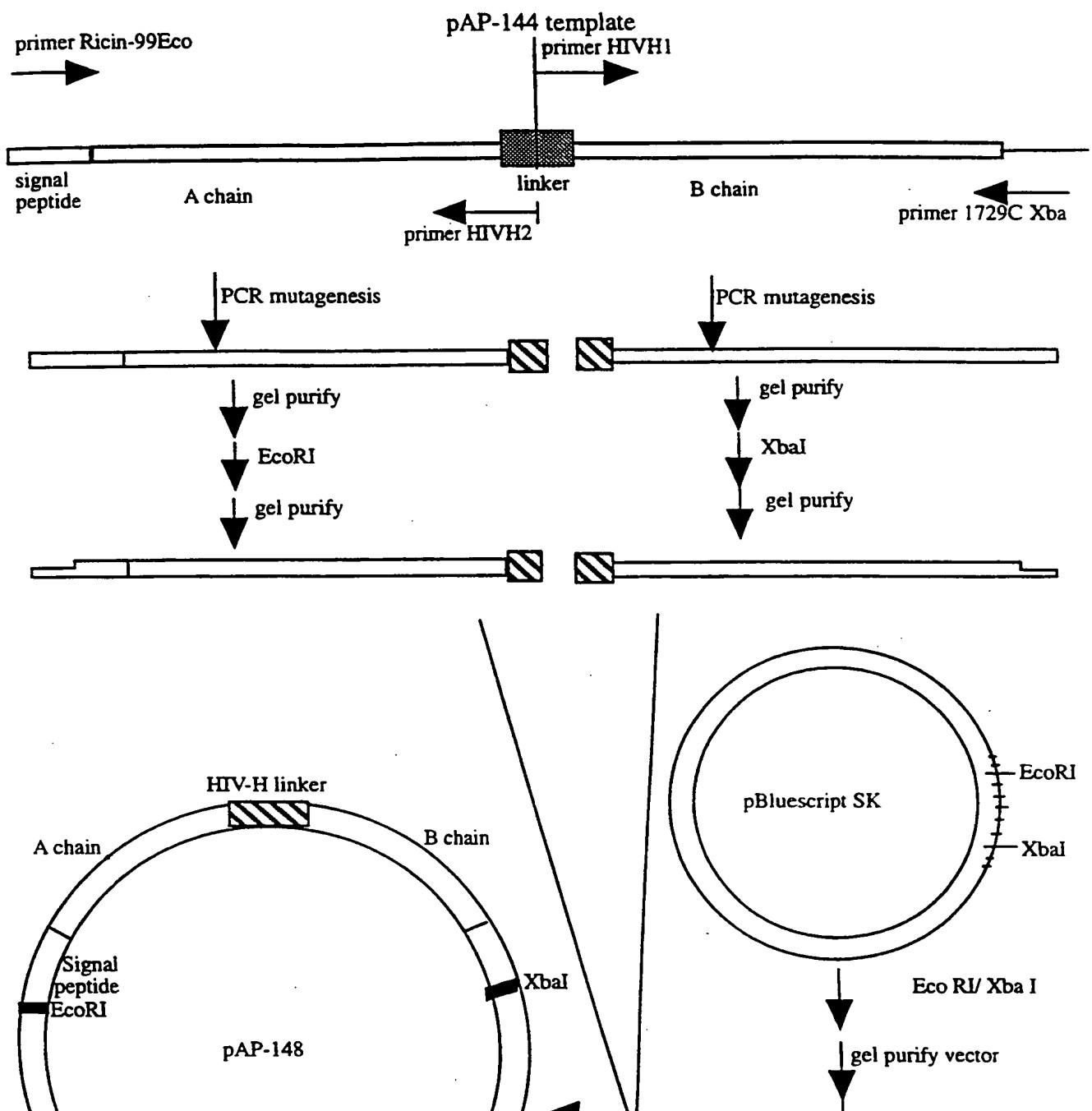
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4/47

FIGURE 2B**WT preprorocin linker**

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5/47
FIGURE 3A

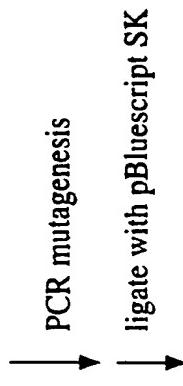
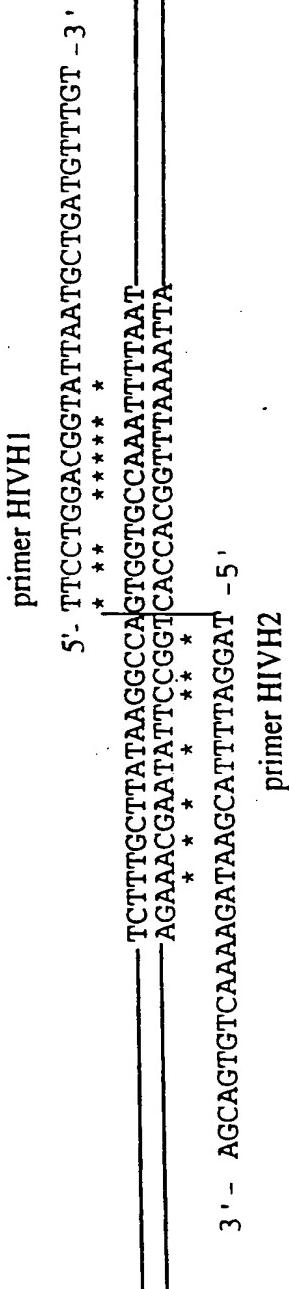


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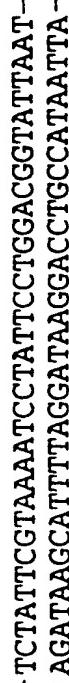
6/47

FIGURE 3B

WT preprorocin linker



pAP-148 linker
(HIV-H variant)



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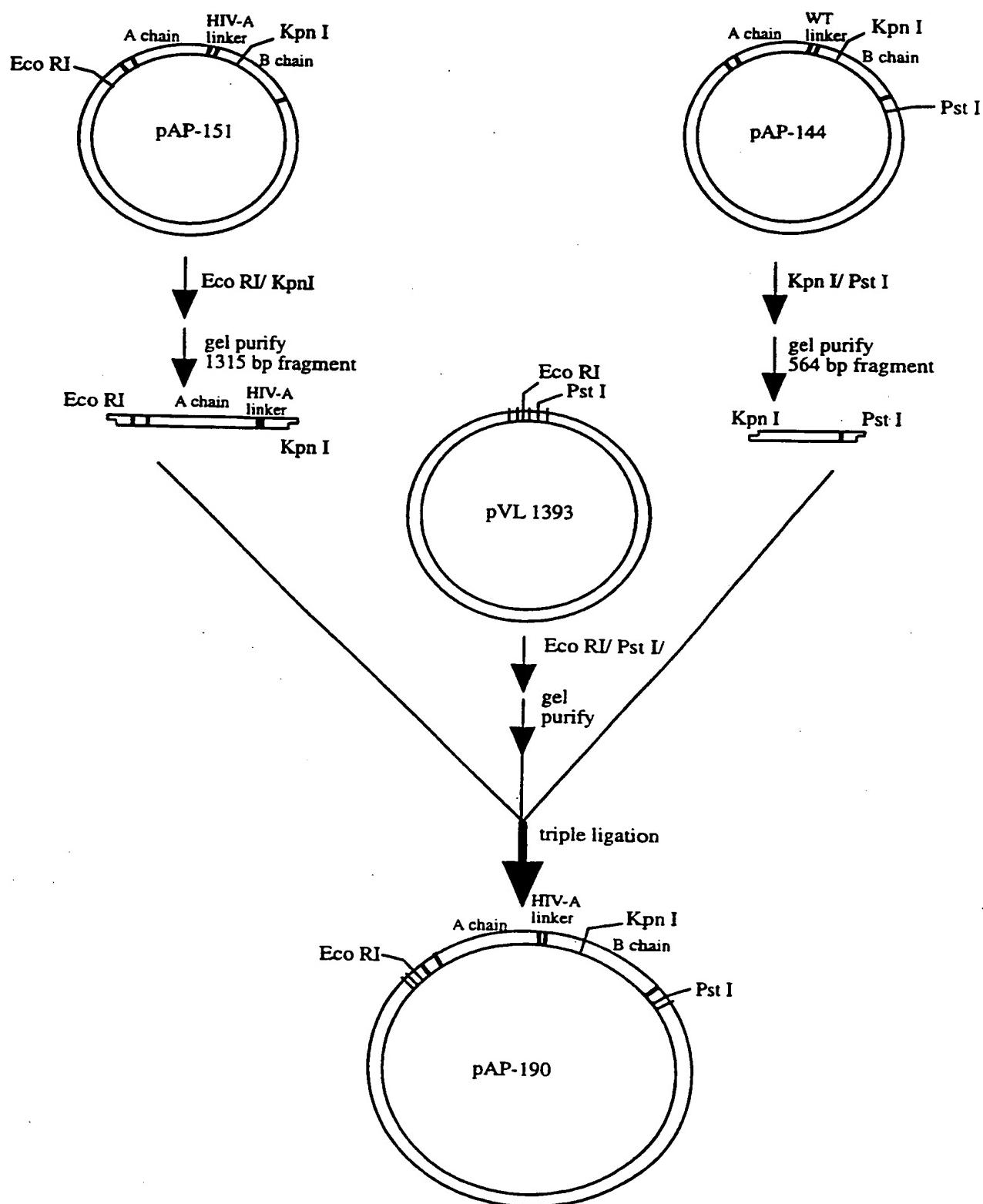
7/47
FIGURE 4

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-146 linker: A chain- V S Q N Y P I V Q N F N -B chain
pAP-147 linker: A chain- S K A R V L A E A M S N -B chain
pAP-148 linker: A chain- S I R K I L F L D G I N -B chain

pAP-146= Ricin cDNA mutant with HIV-A protease linker sequence
pAP-147= Ricin cDNA mutant with HIV-B protease linker sequence
pAP-148= Ricin cDNA mutant with HIV-H protease linker sequence

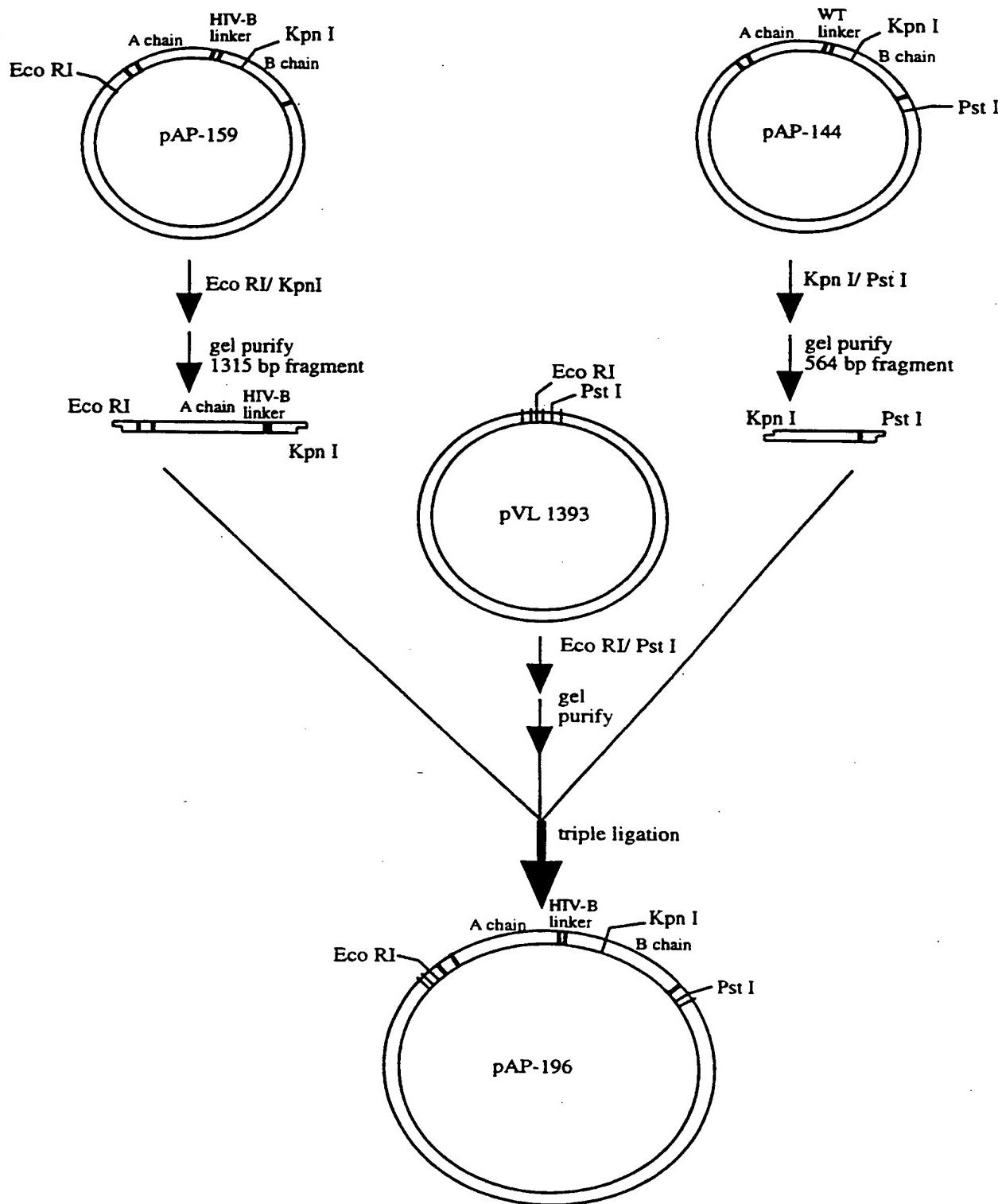
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8/47
FIGURE 5



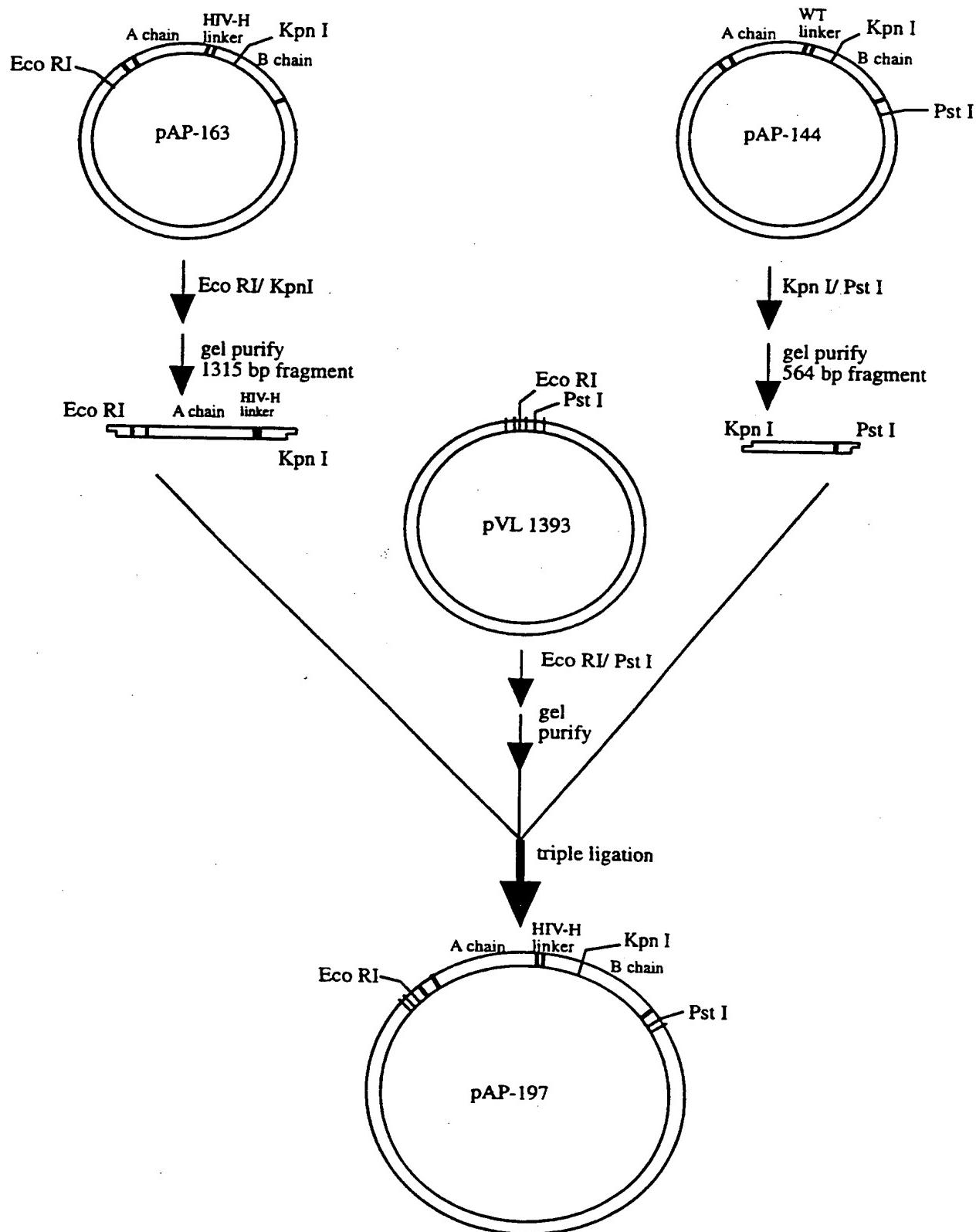
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9/47

FIGURE 6

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10/47
FIGURE 7



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11/47
FIGURE 8

| | 10 | 20 | 30 | 40 | 50 |
|-----|---|----|----|----|----|
| 1 | GAATTCCCTCGAGACGCGTCGACCCGGAGATGAAACCAGGGAGGAAATAC | | | | |
| | CTTAAGGGGAGCTCTGCGCAGCTGGCCTCTACTTGGCCCTCCTTATG | | | | |
| 51 | TATTGTAATATGGATGTATGCAGTGGCAACATGGCTTGGATCCA | | | | |
| | ATAACATTATACTACATACGTACCGTTGACCGAAACAAAACCTAGGT | | | | |
| 101 | CCTCAGGGTGGCTTCACATTAGAGGATAACAACATATTCCCCAACAA | | | | |
| | GGAGTCCCACCAGAAAGTGAATCTCCTATTGTTGTATAAGGGTTGTT | | | | |
| 151 | TACCCAATTATAAACTTACACAGCGGGTGCCACTGTGCAAAGCTACAC | | | | |
| | ATGGGTTAATATTGAAATGGTGTGCCACGGTGACACGTTTCGATGTG | | | | |
| 201 | AAACTTATCAGAGCTGTCGGTCGTTAACAACTGGAGCTGATGTGA | | | | |
| | TTTGAAATAGTCTCGACAAGGCCAGCAAATTGTTGACCTCGACTACACT | | | | |
| 251 | GACATGATATACCACTAGTGTGCAAACAGAGTTGGTTGCCTATAAACCA | | | | |
| | CTGTACTATATGGTCACAACGGTTGTCTCAACCAACGGATATTGGTT | | | | |
| 301 | CGGTTTATTTAGTTGAACCTCTCAAATCATGCAGAGCTTCTGTTACATT | | | | |
| | GCCAAATAAAATCAACTTGAGAGTTAGTACGTCTCGAAAGACAATGTAA | | | | |
| 351 | AGCGCTGGATGTCACCAATGCATATGTGGTCGGTACCGTGCTGGAAATA | | | | |
| | TCGGCACCTACAGTGGTTACGTATAACACCAGCCGATGGCACGACCTTAT | | | | |
| 401 | GCGCATATTTCTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACT | | | | |
| | CGCGTATAAAGAAAGTAGGACTGTTAGTCCTCTACGTCTCGTTAGTGA | | | | |
| 451 | CATCTTTCACTGATGTTCAAATCGATATACATTGCGCTTGGTGGTAA | | | | |
| | GTAGAAAAGTGAACAGTTAGCTATATGTAAGCGGAAACCACCAATT | | | | |
| 501 | TTATGATAGACTGAAACAATTGCTGGTAATCTGAGAGAAAATCGAGT | | | | |
| | AATACTATCTGAACCTGTTGAAACGACCATTAGACTCTCTTTAGCTCA | | | | |
| 551 | TGGGAAATGGTCCACTAGAGGGAGCTATCTAGCGCTTATTACAGT | | | | |
| | ACCCCTTACCAAGGTGATCTCCTCCGATAGACTCGGAAATAATGTCA | | | | |
| 601 | ACTGGTGGCACTCAGCTTCAACTCTGGCTCGTCTTATAATTGCAT | | | | |
| | TGACCACCGTGAGTCGAAGGTTGAGACCGAGCAAGGAATATTAAACGTA | | | | |
| 651 | CCAAATGATTCAGAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGC | | | | |
| | GGTTTACTAAAGTCTTCGTTCTAAGGTTATATAACTCCCTCTTACG | | | | |
| 701 | GCACGAGAATTAGGTACAACCGGAGATCTGCACCAAGATCCTAGCGTAATT | | | | |
| | CGTGCTCTTAATCCATGTTGGCTCTAGACGTGGCTAGGATCGCATTAA | | | | |
| 751 | ACACTTGAGAATTAGTTGGGGGAGACTTCCACTGCAATTCAAGAGTCTAA | | | | |
| | TGTGAACCTTATCAACCCCTCTGAAAGGTGACGTTAGTTCTCAGATT | | | | |
| 801 | CCAAGGAGCCTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTCCA | | | | |
| | GGTTCTCGGAAACGATCAGGTTAAGTTGACGTTCTGCATTACCAAGGT | | | | |
| 851 | AATTCACTGAGTGTGAGTATATTAAATCCCTATCATAGCTCTCATG | | | | |
| | TTAAGTCACACATGCTACACTCATATAATTAGGGATAGTATCGAGAGTAC | | | | |
| 901 | GTGTATAGATGCGCACCTCCACCATCGTCACAGTTGTTCGCAGAACTA | | | | |
| | CACATATCTACCGGTGGAGGTGGTAGCAGTGTCAAACAAAGCGTCTTGAT | | | | |

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12/47

FIGURE 8 (Cont'd)

951 TCCAATAGTCAAATTTAATGCTGATGTTGTATGGATCCTGAGCCCA
 AGGTTATCACGTTAAAATTACGACTACAAACATACTAGGACTCGGGT

 1001 TAGTGCATCGTAGGTCGAAATGGCTATGTTGATGTTAGGGATGGA
 ATCACGCATAGCATCCAGCTTACCGAGATAACAAACTACAATCCCTACCT

 1051 AGATTCCACAACGGAAACGCAATACAGTTGTCGCGATGCAAGTCTAATAC
 TCTAACGGTGTGCTTGCCTATGTCACACCCGGTACGTTAGATTATG

 1101 AGATGCAAATCAGCTCTGGACTTTGAAAAGAGACAATACTATTGATCTA
 TCTACGTTAGTCGAGACCTGAAACTTTCTCTGTTATGATAAGCTAGAT

 1151 ATGGAAAGTGTAACTACTTACGGGTACAGTCGGGAGTCTATGTTGATG
 TACCTTCACAAATTGATGAATGCCCATGTCAGGCCCTCAGATAACACTAC

 1201 ATCTATGATTGCAATACTGCTGCAACTGATGCCACCCGCTGGCAAATATG
 TAGATACTAACGTTATGACGACGTTGACTACGGTGGCGACCCTTATAC

 1251 GGATAATGGAACCATCATAAAATCCCAGATCTAGTCTAGTTAGCAGCGA
 CCTATTACCTGGTAGTATTTAGGGTCTAGATCAGATCAAATCGTCGCT

 1301 CATCAGGGAAAGTGGTACCCACACTTACAGTGCAAAACCAACATTATGCC
 GTAGTCCCTTGTCAACCATGGTGTGAATGTCACGTTGGTTGAAATACGG

 1351 GTTAGTCAAGGTTGGCTTCTACTAATAATACACAACCTTTGTTACAAC
 CAATCAGTCCAACCGAAGGATGATTATTATGTTGGAAAACAATGTTG

 1401 CATTGTTGGCTATATGGTCTGTGCTTGCAAGCAAATAGTGGACAAGTAT
 GTAACAACCGATATACCAGACACGAACGTTGTTATCACCTGTTCAT

 1451 GGATAGAGGACTGTAGCAGTGAAAAGGCTGAACAAACAGTGGCTTTAT
 CCTATCTCTGACATGTCACCTTCCGACTTGTGTCACCCGAGAAATA

 1501 GCAGATGGTCAATACGTCCTCAGCAAACCGAGATAATTGCTTACAAG
 CGTCTACCAAGTTATGCTTGCAAGGAGTCGTTGGCTTACGGAAATGTC

 1551 TGATTCTAATATACGGAAACAGTTGTTAAGATCCTCTTGTGGCCCTG
 ACTAAGATTATATGCCCTTGTCAACAAATTCTAGGAGAGAACACCGGGAC

 1601 CATCCTCTGGCCAACGATGGATGTTCAAGAAATGATGGAACCATTAAAT
 GTAGGAGACCGGTTGCTACCTACAAGTTCTACTACCTGGTAAAATTAA

 1651 TTGTATAGTGGATTGGTGTAGATGTTGAGGGCGATGGATCCGAGCCTTAA
 AACATATCACCTAACCAATCTACACTCCGCTAGCCTAGGCTCGGAATT

 1701 ACAAAATCATTCTTACCCCTCTCCATGGTACCCAAACCAATATGGTTAC
 GTTTAGTAAGAAATGGGAGAGGTACCAACTGGTTGGTTATACCAATG

 1751 CATTATTTGATAGACAGATTACTCTCTGCACTGTTGTCCTGCCAT
 GTAATAAAATCTGTCTAATGAGAGAACGTCACACACACAGGACGGTA

 1801 GAAAATAGATGGCTAAATAAAAAGGACATTGTAATTGTAACGTGAAA
 CTTTTATCTACCGAACATTATTTCTGTAACATTAAACATTGACTTT

 1851 GGACAGCAAGTTATATCGAATTCCCTGCAG
 CCTGTCGTTCAATATAGCTTAAGGACGTC

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13/47
FIGURE 9

| | 10 | 20 | 30 | 40 | 50 |
|-----|---|----|----|----|----|
| 1 | GAATTCCCTCGAGACGCGTCGACCCGGAGATGAAACCGGGAGGAAATAC | | | | |
| | CTTAAGGGGAGCTCTGCGCAGCTGGGCCTACTTTGGCCCTCTTATG | | | | |
| 51 | TATTGTAATATGGATGTATGCAGTGGCAACATGGCTTGATCCA | | | | |
| | ATAACATTATACCTACATACGGTACCGTTGACCGAAACAAACCTAGGT | | | | |
| 101 | CCTCAGGGTGGCTTACATAGAGGATAACAACATATTCCCCAACAA | | | | |
| | GGAGTCCCACCAGAAAGTGTAAATCTCTATTGTTGACAGGGTTGTT | | | | |
| 151 | TACCCAATTATAAACTTTACACAGCGGGGCCACTGTGCAAAGCTACAC | | | | |
| | ATGGGTTAATATTGAAATGGTGTGCCCCACGGTGACACGTTGATGTG | | | | |
| 201 | AAACTTTATCAGAGCTGTCGGTCGTTAACAACTGGAGCTGATGTGA | | | | |
| | TTTGAAATAGTCTCGACAAGCGCCAGCAAATTGTTGACCTCGACTACACT | | | | |
| 251 | GACATGATATAACCAAGTGTGCCAACAGAGTTGGCTTACAAACCA | | | | |
| | CTGTACTATATGGTCACAACGGTTGTCACACCAACGGATATTGGTT | | | | |
| 301 | CGGTTTATTTAGTTGAACCTCTCAAATCATGCAGAGCTTCTGTTACATT | | | | |
| | GCCAAATAAAATCAACTTGAGAGTTAGTACGTCGAAAGACAATGTAA | | | | |
| 351 | AGCGCTGGATGTCACCAATGCATATGTGGTCGGCTACCGTGTGGAAATA | | | | |
| | TCGCGACCTACAGTGGTACGTATAACACCAGCCATGGCACGACCTTAT | | | | |
| 401 | GCGCATATTTCATCCTGACAATCAGGAAGATGCAGAACATCACT | | | | |
| | CGCGTATAAAGAAAGTAGGACTGTTAGTACGTCCTACGTCTCGTTAGTGA | | | | |
| 451 | CATCTTTCACTGATGTTCAAATCGATATACATTGCGTTGGTAA | | | | |
| | GTAGAAAAGTACTACAAGTTAGCTATATGTAAGCGGAAACCACCAATT | | | | |
| 501 | TTATGATAGACTTGAACAATTGCTGGTAATCTGAGAGAAAATATCGAGT | | | | |
| | AATACTATCTGAACCTGTTGAACGACCATTAGACTCTTTATAGCTCA | | | | |
| 551 | TGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTATTACAGT | | | | |
| | ACCCCTTACCAAGGTGATCTCCCTCGATAGACTCGCGAAATAATAATGTCA | | | | |
| 601 | ACTGGTGGCACTCAGCTTCAAACCTGCTCGTCTTATAATTGCGAT | | | | |
| | TGACCAACCGTGAGTCGAAGGTTGAGACCGAGCAAGGAAATTAAACGTA | | | | |
| 651 | CCAAATGATTCAGAAGCAGCAAGATTCAAATATATTGAGGGAGAAATGC | | | | |
| | GGTTTACTAAAGTCTCGTCTAAGGTTATATAACTCCCTTTACG | | | | |
| 701 | GCACGAGAATTAGGTACAACCGGAGATCTGACCAAGATCCTAGCGTAATT | | | | |
| | CGTGCTCTTACATGTTGGCCTCTAGACGTGGCTAGGATCGCATTAA | | | | |
| 751 | ACACTTGAGAATAGTTGGGGAGACTTCCACTGCAATTCAAGAGTCAA | | | | |
| | TGTGAACCTTATCAACCCCTCTGAAAGGTGACGTTAAGTCTCAGATT | | | | |
| 801 | CCAAGGAGCCTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTCCA | | | | |
| | GGTTCCCTCGGAAACGATCAGGTTAAGTGTACGTTCTGCATTACCAAGGT | | | | |
| 851 | AATTCACTGAGTGTGAGTATATAATCCCTATCATAGCTCTCATG | | | | |
| | TTAAGTCACACATGCTACACTCATATAATTAGGGATAGTATCGAGAGTAC | | | | |
| 901 | GTGTATAGATGCGCACCTCCACCATCGTCACAGTTCTAAGGCTCGAGT | | | | |
| | CACATATCTACGCGTGGAGGTAGCAGTGTCAAAAGATTCCGAGCTCA | | | | |
| 951 | GCTAGCGGAGGCAATGTCTAATGCTGATGTTGTATGGATCCTGAGCCCA | | | | |

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14/47
FIGURE 9 (Cont'd)

CGATCGCCTCCGTTACAGATTACGACTACAAACATAACCTAGGACTCGGGT

1001 TAGTCGTATCGTAGGTCGAAATGGCTATGTGTTGATGTTAGGGATGGA
ATCACGCATAGCATCCAGCTTACCAAGATAACACAACATACAAATCCCTACCT

1051 AGATTCCACAAACGGAAACGCAATACAGTTGTCGCCATGCAAGTCTAATAC
TCTAAGGTGTTGCCCTTGCGTTATGTCACACACCAGTACGTTAGATTATG

1101 AGATGCAAATCAGCTCTGGACTTTGAAAAGAGAGACAATACTATTGATCTA
TCTACGTTAGTCGAGACCTGAAACTTTCTCTGTTATGATAAGCTAGAT

1151 ATGGAAAGTGTAACTACTTACGGGTACAGTCCGGGAGTCTATGTGATG
TACCTTCACAAATTGATGAATGCCATGTCAGGCCCTCAGATAACACTAC

1201 ATCTATGATTGCAAACTACTGCTGCAACTGATGCCACCCGCTGGCAAATATG
TAGATACTAACGTTATGACGACGTTGACTACGGTGGCGACCGTTATAC

1251 GGATAATGGAACCATCATAAATCCCAGATCTAGTCTAGTTAGCAGCGA
CCTATTACCTTGGTAGTATTAGGGCTAGATCAGATCAAATCGTCGCT

1301 CATCAGGGAACAGTGGTACACACTTACAGTGCAAACCAACATTATGCC
GTAGTCCTTGTCAACATGGTGTGAATGTCACGTTGGTTGTAAATACGG

1351 GTTAGTCAGGTTGGCTTCTACTAATAATACACAACCTTTGTTACAC
CAATCAGTTCCAACCGAAGGATGATTATTATGTTGGAAAACAATGTTG

1401 CATTGTTGGCTATATGGCTGTGCTGCAAGCAAATAGTGGACAAGTAT
GTAACAACCCGATATACCAAGACACGAACGTTGTTATCACCTGTTCAT

1451 GGATAGAGGACTGTAGCAGTGAAAAGGCTGAACAAACAGTGGCTTTAT
CCTATCTCCTGACATCGTCACTTTCCGACTTGTGTCACCCGAGAAATA

1501 GCAGATGGTTCAATACGTCTCAGAAAACCGAGATAATTGCCCTAACAG
CGTCTACCAAGTTATGCGAGGCTGTTGGCTCTATTAACGGAATGTT

1551 TGATTCTAATATACGGAAACAGTTGTTAAGATCCTCTCTGTCGGCCCTG
ACTAAGATTATATGCCCTTGTCAACAAATTCTAGGAGAGAACACCGGGAC

1601 CATCCTCTGGCCAACGATGGATGTTCAAGAAATGATGGAACCATTAAAT
GTAGGAGACCGGTTGCTACCTACAAGTTCTTACCTGGTAAATTTA

1651 TTGTATAGTGGATTGGTGTAGATGTGAGGCGATCGGATCCGAGCCTTAA
AACATATCACCTAACACAAATCTACACTCCGCTAGCCTAGGCTCGGAATT

1701 ACAAAATCATTCTTACCCCTCTCCATGGTGACCCAAACCAAATATGTTAC
TGTGTTAGTAAGAAATGGGAGAGGTACCACTGGGTTGGTTATACCAATG

1751 CATTATTTGATAGACAGATTACTCTTGCAGTGTGTTGTCCTGCCAT
GTAATAAAACTATCTGTCTAATGAGAGAACGTCACACACAGGACGGTA

1801 GAAAATAGATGGCTTAAATAAAAGGACATTGAAATTTGTAACTGAAA
CTTTTATCTACCGAATTATTTCTGTAACATTAAAACATTGACTTT

1851 GGACAGCAAGTTATATCGAATTCTGCAG
CCTGTCGTTCAATATAGCTTAAGGACGTC

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15/47

FIGURE 10

10 20 30 40 50

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1  GAATTCCCTCGAGACCGCGTCGACCCGGAGATGAAACCGGGAGGAAATAC
   CTTAAGGGGAGCTCTGCCAGCTGGCCTCTACTTGGCCCTCCTTATG

51  TATTGTAATATGGATGTATGCAGTGGCAACATGGCTTGTGATCCA
   ATAACATTATACTACATACGTACCAGTGTACCGAAACAAACCTAGGT

101 CCTCAGGGTGGCTTACATTAGAGGATAACAACATATTCCCCAAACAA
   GGAGTCCCACCAAGAAAGTGAATCTCCTATTGTTGATAAGGGGTTGTT

151 TACCCAATTATAAACTTTACACAGCGGGTGCCACTGTGCAAAGCTACAC
   ATGGGTTAATATTGAAATGGTGTGCCAACGGTGACACGTTCGATGTG

201 AAACTTATCAGAGCTGTTCGCGGTCGTTAACAACTGGAGCTGATGTGA
   TTTGAAATAGTCTCGACAAGGCCAGCAAATTGTTGACCTCGACTACACT

251 GACATGATATAACAGTGGTGCACAAACAGAGTTGGCTTGCCTATAAACCAA
   CTGTACTATATGGTCACACGGTTGTCTCAACCAAACGGATAATTGGTT

301 CGGTTTATTAGTTGAACTCTCAAATCATGCAGAGCTTCTGTTACATT
   GCCAAATAAAATCAAATTGAGAGTTAGTACGTCTCGAAAGACAATGTAA

351 AGCGCTGGATGTCACCAATGCATATGGTGGCTACCGTGCTGGAAATA
   TCGCGACCTACAGTGGTACGTATACACCAGCCATGGCACGACCTTAT

401 GCGCATATTCTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACT
   CGCGTATAAAGAAAAGTAGGACTGTTAGTCCTCTACGTCTCGTTAGTGA

451 CATCTTTCACTGATGTTCAAAATCGATATACATTGGCTTGGTAA
   GTAGAAAAGTGAATACAAGTTAGCTATATGTAAGCGGAAACCACCAATT

501 TTATGATAGACTTGAACAACCTGGTGGTAATCTGAGAGAAAATATCGAGT
   AATACTATCTGAACCTGTTGAAACGACCATTAGACTCTTTATAGCTCA

551 TGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTATTACAGT
   ACCCTTACCAAGGTGATCTCCTCCGATAGAGTCGCGAAATAATAATGTCA

601 ACTGGTGGCACTCAGCTTCAACTCTGGCTCGTTCTTATAATTGCAT
   TGACCACCGTGAGTCGAAGGTTGAGACCGAGCAAGGAAATTAAACGTA

651 CCAAATGATTCAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGC
   GGTTTACTAAAGTCTCGTCTAAGGTTATATAACTCCCTTTACG

701 GCACGAGAATTAGGTACAACCGGAGATCTGCACCAAGATCCTAGCGTAATT
   CGTGTCTTAATCCATGTTGGCCTCTAGACGTGGCTAGGATCGCATTAA

751 ACACTTGAGAATAGTTGGGGAGACTTCCACTGCAATTCAAGAGTCTAA
   TGTGAACCTTATCAACCCCTCTGAAAGGTGACGTTAAGTCTCAGATT

801 CCAAGGAGCCTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTCCA
   GGTTCTCGAAACGATCAGGTTAAGTGTACGTTCTGCATTACCAAGGT

851 AATTCACTGTCAGATGTGAGTATATTAAATCCCTATCATAGCTCTCATG
   TTAAGTCACACATGCTACACTCATATAATTAGGGATAGTATCGAGAGTAC

901 GTGTATAGATGCGCACCTCCACCACGTACAGTTCTATTGTAAGTAA
   CACATATCTACCGTGAGGTGGTAGCAGTGTCAAAGATAAGCATTAA

951 CCTATTCTGGACGGTATTAATGCTGATGTTGTATGGATCCTGAGCCCA

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16/47

FIGURE 10 (Cont'd)

GGATAAGGACCTGCCATAATTACGACTACAAACATACCTAGGACTCGGGT

1001 TAGTCGTATCGTAGGTCGAAATGGCTATGTGTGATGTTAGGGATGGA
ATCACGCATAGCATCCAGCTTACCACTACAACTACAATCCCTACCT

1051 AGATTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATAC
TCTAAGGTGTTGCCCTTGCCTTATGCAACACCAGTACGTTAGATTATG

1101 AGATGCAAATCAGCTCTGGACTTTGAAAAGAGACAATACTATTGATCTA
TCTACGTTAGTCGAGACCTGAAACTTTCTCTGTTATGATAAGCTAGAT

1151 ATGGAAAGTGTAACTACTTACGGGTACAGTCCGGGAGTCTATGTGATG
TACCTTCACAAATTGATGAATGCCCATGTCAGGCCCTCAGATACTAC

1201 ATCTATGATTGCAAACTACTGCTGCAACTGATGCCACCCGCTGGCAAATATG
TAGATACTAACGTTATGACGACGTTGACTACGGTGGCGACCGTTATAC

1251 GGATAATGGAACCATCATAAATCCCAGATCTAGTCTAGTTAGCAGCGA
CCTATTACCTGGTAGTTAGGGTCTAGATCAGATCAAATCGTCGCT

1301 CATCAGGGAACAGTGGTACACACTTACAGTGCAAACCAACATTATGCC
GTAGTCCTGTCACCATGGTGTGAATGTCACGTTGGTTGAAATACGG

1351 GTTAGTCAAGGTTGGCTTCTACTAATAATACACAACCTTTGTTACAAC
CAATCAGTTCCAACCGAAGGGTATTATGTGTTGGAAAACAATGTTG

1401 CATTGTTGGGCTATATGGTCTGTGCTGCAAGCAAATAGTGGACAAGTAT
GTAACAACCGATATACCAGACACGAACGTTGTTATCACCTGTTACA

1451 GGATAGAGGACTGTAGCAGTAAAAGGCTGAACAACAGTGGCTTTAT
CCTATCTCCTGACATCGTCACCTTCCGACTGTTGTCACCCGAGAAATA

1501 GCAGATGGTTCAATACTGCTCAGCAAAACCGAGATAATTGCCCTTACAAG
CGTCTACCAAGTTATGCAAGGAGTCGTTGGCTCTATTACCGGAATGTT

1551 TGATTCTAATATACGGGAAACAGTTGTTAAGATCCTCTTGTGGCCCTG
ACTAAGATTATATGCCCTTGTCAACAATTCTAGGAGAGAACACCGGGAC

1601 CATCCTCTGGCCAACGATGGATGTTCAAGAATGATGGAACCATTAAAT
GTAGGAGACCGGTTGCTACCTACAAGTTACTACCTGGTAAATTTA

1651 TTGTATAGTGGATTGGTGTAGATGTGAGGGGATCGGATCCGAGCCTAA
AACATATCACCTAACCAATCTACACTCCGCTAGCCTAGGCTCGGAATT

1701 ACAAAATCATTCTTACCCCTCTCCATGGTGAACCAAACCAAATATGGTTAC
TGTGTTAGTAAGAAATGGGAGAGGTACCAACTGGGTTGGTTATACCAATG

1751 CATTATTTGATAGACAGATTACTCTTGCAGTGTGTGTCCTGCCAT
GTAATAAAACTATCTGTCTAATGAGAGAACGTCACACACAGGACGGTA

1801 GAAAATAGATGGCTTAAATAAAAGGACATTGTAATTGTAACGTGAAA
CTTTTATCTACCGAATTATTTTCTGTAACATTAAAACATTGACTTT

1851 GGACAGCAAGTTATCGAATCCTGCA
CCTGTCGTTCAATATAGCTTAAGGACGTC

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17/47

FIGURE 11

ID PVL1393 preliminary; circular DNA; SYN;
9632 BP.
XX
AC IG1137;
XX
DT 01-FEB-1993 (Rel. 7, Created)
DT 01-JUL-1995 (Rel. 12, Last updated, Version
1)
XX
DE E. coli plasmid vector pVL1393 - complete.
XX
KW cloning vector.
XX
OS Cloning vector
OC Artificial sequences; Cloning vehicles.
XX
RN [1]
RC p2Bac from baculovirus
RC p2Blue from p2Bac
RC pBlueBac from AcNPV
RC pBlueBac2 from AcNPV
RC pBlueBacIII from AcNPV
RC pBlueBacHisA from AcNPV
RC pBlueBacHisB from AcNPV
RC pBlueBacHisC from AcNPV
RC pVL1392, pVL1393 from pAc360
RA ;
RT ;
RL The Digest 5:2-2(1992).
XX
CC NM (pVL1393)
CC CM (yes)
CC NA (ds-DNA)
CC TP (circular)
CC ST ()
CC TY (plasmid)
CC SP (British
Biotechnology) (Invitrogen)
CC HO (E.coli NM522) (E.coli
INValphaF') (insect)
CC CP ()
CC FN (expression) (transfer)
CC SE ()
CC PA (pAC360)
CC BR (pVL1392)
CC OF ()
CC OR ()
XX
FH Key Location/Qualifiers
FH

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18/47

FIGURE 11 (Cont'd)

FT misc_feature 0..0
 FT /note="1. pAc360, ori/amp/AcMNPV
 polyhedrin gene
 FT transposon -> pVL1393 9632bp"
 0..0
 FT /note="TRN AcMNPV"
 misc_binding 868..868
 FT /note="SIT SacII"
 misc_binding 1395..1395
 FT /note="SIT ApaI"
 misc_binding 1901..1901
 FT /note="SIT XhoI"
 promoter 0..0
 FT /note="PRO AcMNPV polyhedrin gene"
 misc_binding 0..0
 FT /note="MCS
 FT BamHI-SmaI-XbaI-EcoRI-NotI-XmaIII-PstI-
 BglII"
 FT rep_origin 0..0
 FT /note="ORI E. coli pMB1 (ColE1 and
 pBR322)"
 FT CDS complement(0..0)
 FT (bla) /note="ANT E. coli beta-lactamase gene
 FT XX ampicillin resistance gene (apr/amp)"
 SQ Sequence 9632 BP; 2602 A; 2122 C; 2176 G; 2732 T; 0
 other;
 aagctttact cgtaaagcga gttgaaggat catathtagt tgcgtttatg
 agataagatt gaaagcacgt gtaaaaatgtt tcccgccgt tggcacaact
 atttacaatg cggccaagtt ataaaagatt ctaatctgat atgttttaaa
 acaccttgcgca ggcccggagtt gtttgcgtac gtgactagcg aagaagatgt
 gtggaccgcga gaacagatag taaaacaaaa ccctagtatt ggagcaataa
 tcgatttaac caaacacgtct aaatattatg atggtgtgca tttttgcgg
 gcgggcctgt tataaaaaaa aattcaagta cctggccaga ctttgccgcc
 tgaaagcata gttcaagaat ttattgacac ggtaaaaagaa tttacagaaaa
 agtgtcccgcatgttggtg ggcgtgcact gcacacacgg tattaatcgc
 accggttaca tggtgtgcag atatttaatg cacaccctgg gtattgcgcc
 gcaggaagcc atagatagat tcgaaaaaagc cagaggtcac aaaattgaaa
 gacaaaatttgcgtcaagat ttattaaattt aattaatatt atttgcattc
 tttaacaaat actttatcct atttcaaattt tggtgcgtt cttccagcga
 accaaaacta tgcttcgtt gctccgttta gcttgtagcc gatcagtggc
 gttgttccaa tcgacggtag gattaggccg gatattctcc accacaatgt
 tggcaacgtt gatgttacgt ttatgtttt ggtttccac gtacgtctt
 tggccggtaa tagccgtaaa cgtatgtccg tcgcgcgtca cgcacaacac
 cggatgtttcgcttgcgttgcgttccg cgggggtattt aaccgcgcga tccgacaaat
 ccaccacttggcaactaaa tcggtgacct ggcgtctttt ttctgcatt
 atttcgtctt tctttgcattt ggtttccat ggtttccat ggtttccac
 tagatcagtc atgacgcgcg tgacctgcaaa atctttggcc tcgatctgct
 tgccttgat ggcaacgatg cgttcaataa actcttggtt tttaacaatgt
 tcctcggtt tttgcgttccac caccgcttgc agcgcgttgc tgcgtctgg
 gaatgtcgca atcagcttag tcaccaactg tttgcgttcc tcctccgtt
 gtttgatcgcc gggatcgatc ttgcgttccac agagcacttgc aggaattact
 tcttctaaaa gccattcttg taattctatg gcgtaaggca atttggactt

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19/47

FIGURE 11 (Cont'd)

cataatcagc tgaatcacgc cggatttagt aatgagcaact gtatgcggct
gcaaatacag cgggtcgccc ctttacacga cgctgttaga ggtaggccc
ccattttgga tggctcgctc aaataacgat ttgtatttat tgtctacatg
aacacgtata gctttatcac aaactgtata tttaaactg ttagcgacgt
ccttggccac gaaccggacc tgggtcgac gctctagcac gtaccgcagg
ttgaacgtat cttctccaaa tttaaattct ccaattttaa cgcgagccat
tttgatacac gtgtgtcgat tttgcaacaa ctattgttt ttaacgcaaa
ctaaacttat tgggtaaagc aataattaaa tatggggaa catgcgccgc
tacaacactc gtcgttatga acgcagacgg cgccggtctc ggcgcaagcg
gctaaacgt gttgcgcgtt atttgcatat caacgcggca aacatcgcaa aagccaatag
tacagtttg atgacgccta taacggcgat ttttaatt atcttattta
ataaaatagt tcgttgacgc caactccccg cccgcgttga ctcgctgcac
ctcgagcagt gaccagcggc gtcgcgcacg gtggccgaac acgtcgagcg
ggtgtcgat tagtgcgatt aggcacgtcg gcctccaagt ggcaatattg
accgaatgat gtacgtccga acgttgcattt gcatgcaagc cgaaattaaa
gcaaattcga aaatatatac aaaacgttgc acatcctcgc tttaatcat
cgtnnnncat tagtgcgatt aaatcgcgc aatcgagtc aaatcgatcaac
tcatttgcgat gtattcccga gtcaagcgca ggcgttgc taacaaacta
gccatcttgt aagttagttt catttaatgc aactttatcc aataatata
tatgtatcgc acgtcaagaa ttaacaatgc gcccgttgc gcatctcaac
acgactatga tagagatcaa ataaagcg aattaaatag cttgcacgc
aacgtgcacg atctgtgcac gcgttccggc acgagcttg attgtaataa
gttttacga agcgatgaca tgaccccgat agtgacaacg atcacgccc
aaagaactgc cgactacaaa attaccgagt atgtcggta cgttaaaact
attaagccat ccaatcgacc gttagtcgaa ttaggaccgc tggtgcgaga
agccgcgaag tatggcgaat gcatcgata acgtgtggag tccgctcatt
agagcgtcat gtttagacaa gaaagctaca tatttaattt atcccgtatga
tttatttgcgaa aaattgaccc taactccata cacggtattc tacaatggcg
gggttttgcggt caaaatttcc ggactgcgat tgtacatgct gtaacggct
ccgccccacta ttaatgaaat taaaattcc aattttaaaa aacgcagcaa
gagaaacatt tttatgtacaaat gatcgatc aatgcgtaga aggaaagaaa
acatgctgaa caacaagatt aatatgcctc cgtgtataaa aaaaatattg
aacgatttga aagaaaacaa tgtatgtacaaat gttaccgcgc ggcggtatgt
gtttatacta aactgttaca tttatgtacaaat gctctgacgc atttctacaa
aaaaccgatg ttaatcaag gtcaaaacat gcatctttt atcaaatccc
aagtgtgtgg taaaccacca aactgcacaa ctcaatccta tttgtatc
ttgctggcaa ctgcaagggt aatgctaaa tttgtttt attaacgata
aaacaattat aatgttagt catttgcgat attatctata attgaaaacg
gcaacaagaa catttgcgat aatcatttc aatgtattca cgtagttata
atcgctgagg taatattaa aatcatttc aatgtattca cgttaattt
gcfgacaatat aattttattt aatcatttc aatgtattca cgttaattt
tcttcgtatt ctttctcttt aatgttagt aatcatttc aatgtattca
gttattatcg tatccatata ttttaatgg ggtgtatagt accgctgcgc
tgtcataaat atatatgtct ttaatttata aacagtgtca tttctgtat
atagttttc tggatattac aattttata aatgttagt aatcatttc
gtgtgttgct ttaatttata aatgttagt aatcatttc aatgtattca
cggtttgtat caatatgtt cttctagttca ccggcatagt
acgcagcttc acataacttt ccaaaatgtt ttcttagttca
tccctttct atactattgt ccggcatagt
cagccattgt aatgagacgc acaaaactaat atcacaact
aatcatttc aatgttagt aatcatttc aatgtattca
tttttagcag caccggattt
gtacgaaccg taaaacaaaa acagttcacc
ctgcgagcag ttgtttgtt taaaaataaa
acaactaat atcacaact
ggaaatgtct

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20/47
FIGURE 11 (Cont'd)

ctgtcccgat ttatttgaaa cactacaaat taaaggcag ctttcgtacc
 aacttggtag caatatttatt agacagctgt gtgaagcgct caacgatttg
 cacaaggcaca atttcataca caacgacata aaactcgaaa atgtcttata
 tttcgaagca cttgatcgcg tgtatgtttg cgattacgga ttgtgcaaac
 acgaaaactc acttagcggt cacgacggca cggtggagta ttttagtccg
 gaaaaaaattc gacacacaac tatgcacgtt tcgtttgact ggtacgcggc
 gtgttaacat acaagttgct aacgtaatca tggtcatagc tggttcctgt
 gtgaaattgt tatccgctca caattccaca caacatacga gccggaagca
 taaagtgtaa agcctgggt gcctaattgag ttagcttaact cacattaatt
 gcgttgcgt cactgcccgc tttccagtcg gaaaacctgt cgtgccagct
 gcattaaatga atcggccaac ggcggggag aggccggttg cgtattggc
 gctcttcgc ttcctcgctc actgactcg ctcgctcgt cgttcggctg
 cggcgagcgg tatcagctca ctcaaaaggcg gtaatacgtt tatccacaga
 atcagggat aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg
 ccaggaaccg taaaaaggcc gcgttgctgg cgttttcca taggctccgc
 cccccgtacg agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa
 cccgacagga ctataaaagat accaggcggtt tccccctgga agctccctcg
 tgcgctctcc tggtccgacc ctgcccgtt ccggataacct gtccgcctt
 ctcccttcgg gaagcgtggc gctttctcat agtcacgct gtaggtatct
 cagttcggtg tagtgcgtt gctccaagct ggctgtgtg cacgaacccc
 ccgttcagcc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc
 aaccggtaa
 gacacgactt atcgccactg gcagcagccca ctggtaacag gattagcaga
 gcgaggatag taggcgggtgc tacagagttc ttgaagtggt ggcttaacta
 cggctacact agaaggacag tattttgtat ctgcgctctg ctgaagccag
 ttaccttcgg aaaaagagtt ggtagctttt gatccggcaa acaaaccacc
 gctggtagcg gtgggttttt tggttgcaag cagcagatta cgcgcagaaa
 aaaaggatct caagaagatc ctttgatctt ttctacgggg tctgacgctc
 agtggAACGA aaactcacgt taagggattt tggtcatgag attatcaaaa
 aggatcttca cctagatcct tttaaatcaa aaatgaagtt tttaaatcaat
 ctaaaagtata tatgagtaaa ctgggtctga cagttaccaa tgcttaatca
 gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc
 tgactccccg tcgttagat aactacgata cgggagggtt taccatctgg
 cccccagtgc gcaatgatac cgcgagaccc acgctcaccc gctccagatt
 tatcagcaat aaaccagccca gccggaaaggg ccgagcgcag aagtggcct
 gcaactttat ccgcctccat ccagtctatt aattgttgcc gggaaagctag
 agtaagtagt tcgcccgtt aatgtttgcg caacgttggt gccattgcta
 caggcatcgt ggtgtcacgc tcgtcggtt gtagggcttc attcagctcc
 ggttcccaac gatcaaggcg agttacatga tccccatgt tttggaaaa
 agcggtagc tccttcggc ctcggatcgat tttttttttt aagttggccg
 cagtgttatac actcatggtt atggcagcac tgcataattc tcttactgtc
 atgccatccg taagatgctt ttctgtgact ggtgagttact caaccaagtc
 attctgagaa tagtgtatgc ggcgaccggag ttgtcttgc ccggcgtaa
 tacggataa taccgcgcca catagcagaa cttaaaagt gctcatcatt
 ggaaaaacgtt ctccggggcg aaaactctca aggatcttac cgctgttgag
 atccagttcg atgtacccca ctcgtgcacc caactgatct tcagcatctt
 ttactttcac cagcggttct gggtagccaa aaacaggaag gcaaaatgcc
 gcaaaaaagg gaataaggc gacacggaaa tttttttttt tttttttttt
 ccttttcaa tattattgaa gcatattatca gggttattgt ctcatgagcg
 gatacatatt tgaatgtatt tagaaaaata aacaaatagg gttccggcgc
 acatttcccc gaaaagtgc acctgacgtc taagaaacca ttattatcat
 gacattaacc tataaaaaata ggcgtatcac gaggccctt cgttcgcgc
 gtttcggta tgacggtgaa aacctctgac acatgcagct cccggagacg
 gtcacagctt gtcgtaaagc ggtgcccggg agcagacaag cccgtcagg

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21 / 47

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22/47

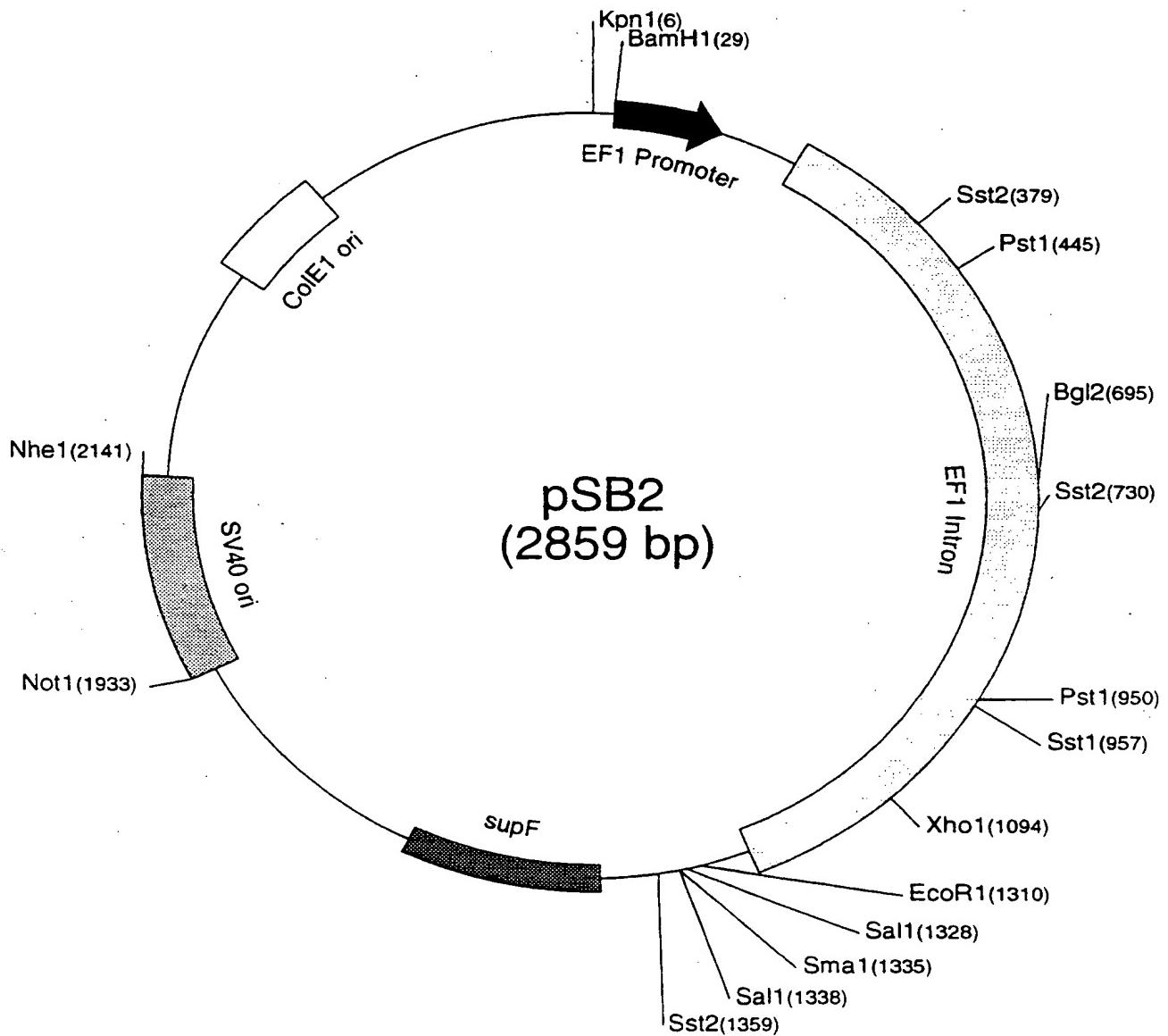
FIGURE 11 (Cont'd)

cgcgtcagcg ggtgttggcg ggtgtcgaaa ctggcttaac tatgcggcat
cagagcagat tgtactgaga gtgcaccata tgcggtgtga aataccgcac
agatgcgtaa ggagaaaata ccgcattcagg cgccatttcgc cattcaggt
gcaactgt tggaaaggc gatcggtgcg ggctttcg ctattacgcc
agctggcgaa agggggatgt gctgcaaggc gattaagttg ggtaacgcca
gggtttccc agtcacgacg ttgtaaaacg acggccagtg cc

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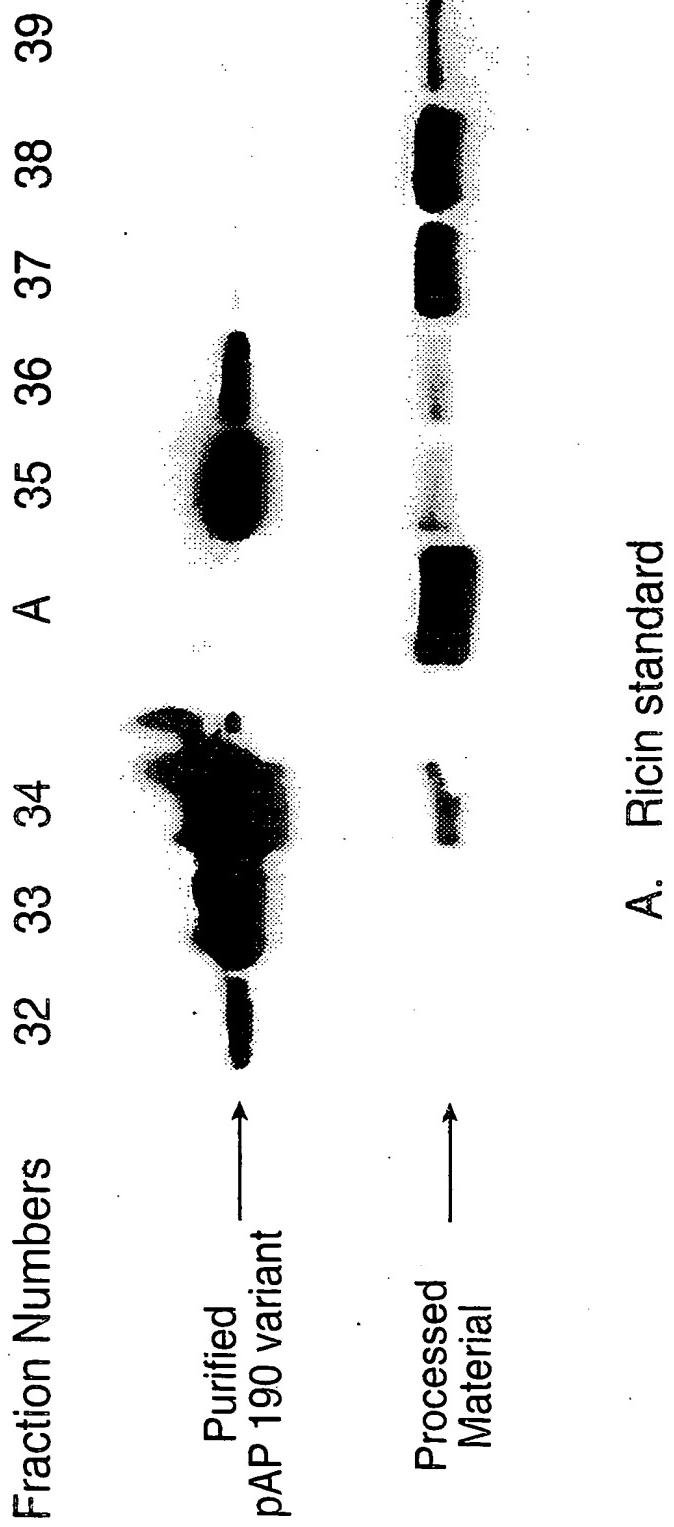
23/47

FIGURE 12

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24/47

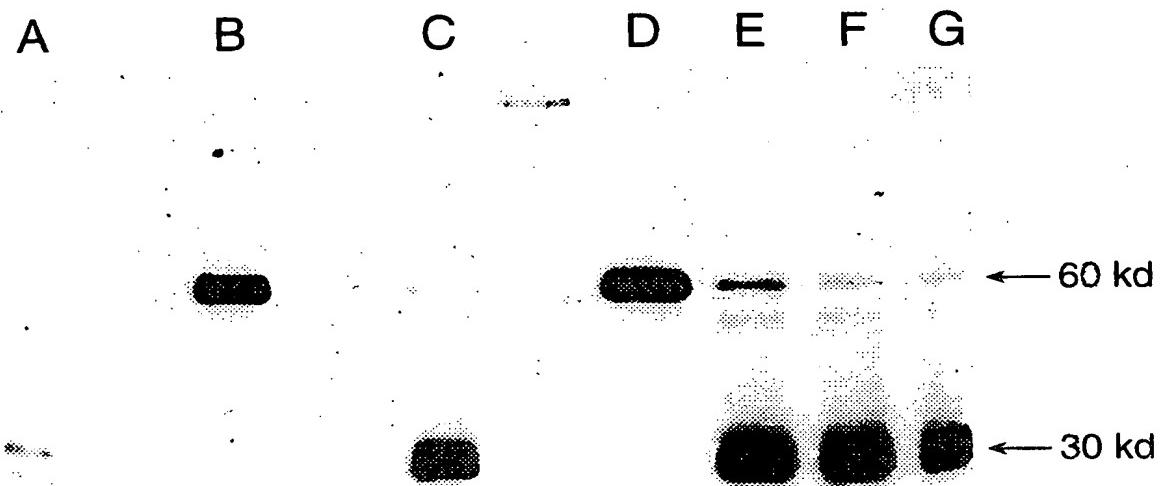
FIGURE 13
Confirmation of pAP 190 purity by
Western analysis



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FIGURE 14

Cleavage of pAP 190 by HIV protease

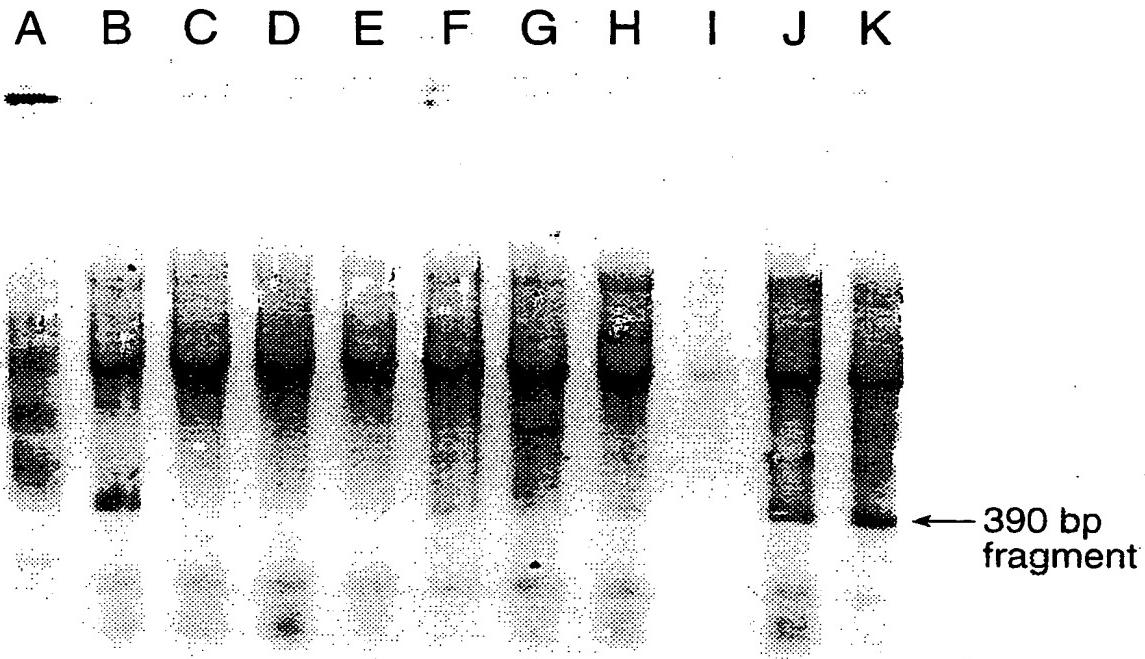


- A. Ricin standard
- B. pAP 190
- C. pAP 190 + HIV protease (3 hours)
- D. pAP 190
- E. pAP 190 + HIV protease (30 minutes)
- F. pAP 190 + HIV protease (1 hour)
- G. pAP 190 + HIV protease (2 hours)

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FIGURE 15

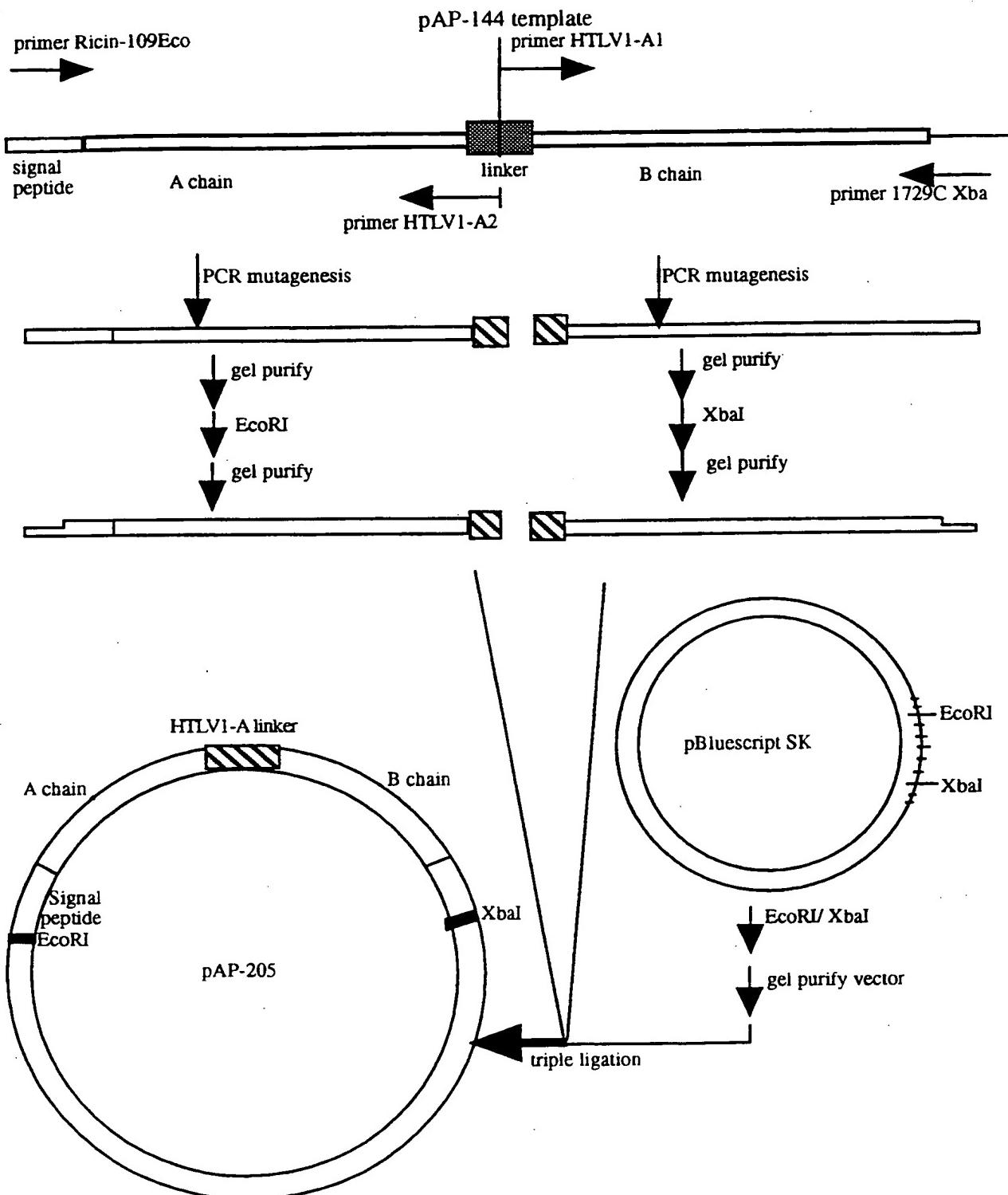
Activation of pAP 190



- A. RNA Ladder
- B. Ricin A chain
- C. Negative control
- D. 340 pg pAP 190 variant
- E. 2.1 ng pAP 190 variant
- F. 12.5 ng pAP 190 variant
- G. 75 ng pAP 190 variant
- H. 340 pg 190 + HIV protease
- I. 2.1 ng 190 + HIV protease
- J. 12.5 ng 190 + HIV protease
- K. 75 ng 190 + HIV protease

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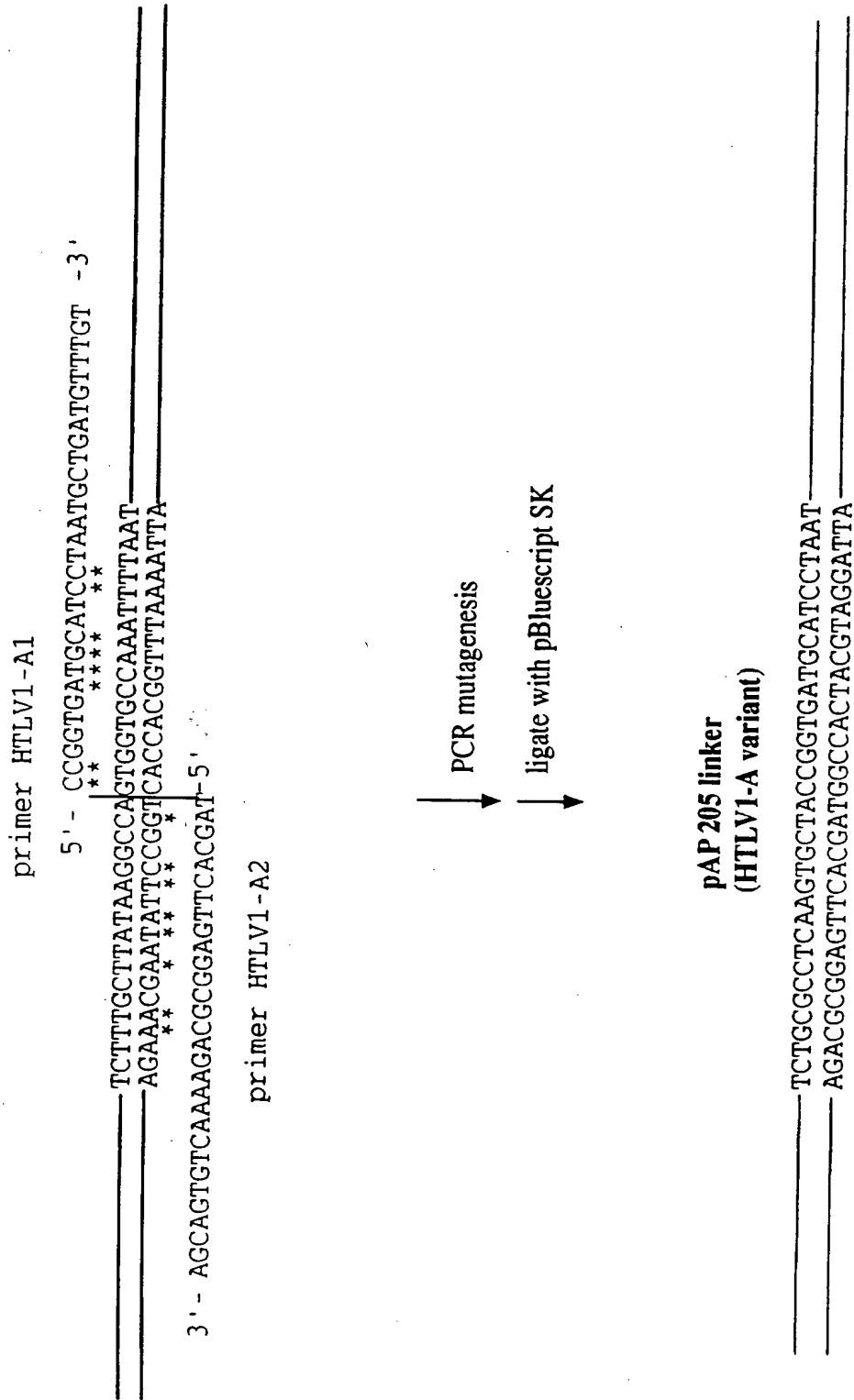
27/47

FIGURE 16A

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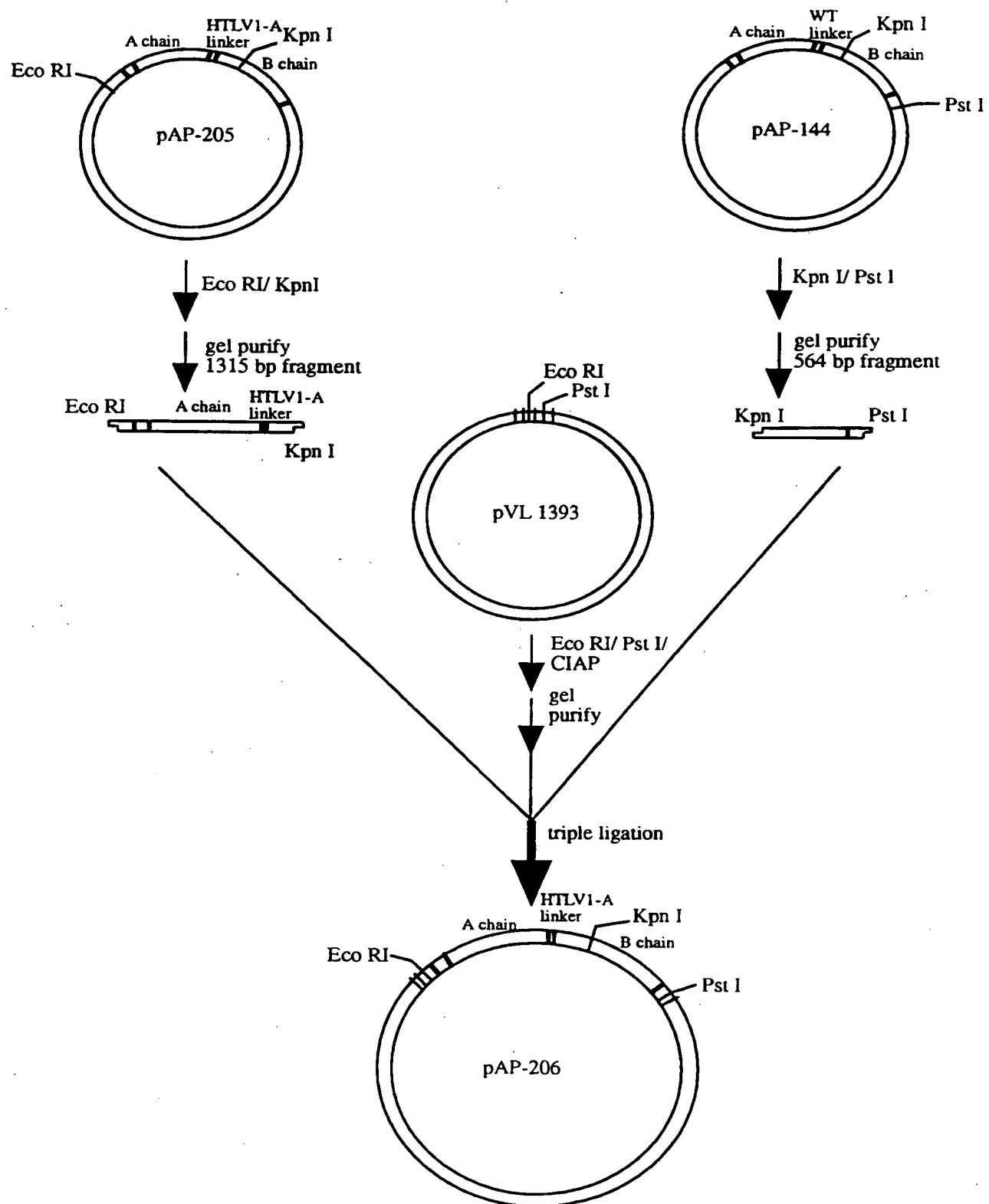
FIGURE 16B

WT preprorocin linker



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29/47
FIGURE 16C



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30/47

FIGURE 16D

10 20 30 40 50

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1  GAATTCATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTGGCCCTCCTTATGATAACATTACACATACGTCA

51  GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTCACATTAG
   CGGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAAGTGTAA

101 AGGATAACAACATATTCCCCAAACAATACCAATTATAAACCTTACCA
    ACCTATTGTTGATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACCTTATCAGAGCTGTTGCCG
   CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTGACAAGGCC

201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCA
   TGTCTCAACCAAACGGATAATTGGTTGCCAAATAAAACTCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAACTCATCTTCACTGATGTTAAAAT
   TAGTCCTTCTACGTCTCGTTAGTGAAGTAGAAAGTGAACACAAGTTTA

451 CGATATACATTGCCTTGGGTGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCAACCATTAATAACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTTTATAGCTCAACCCCTTACCGAGTGTATCCTCC

551 CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCTTATAATTGATCCAAATGATTTCAGAACAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTTTACCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
   CTAGACGTGGTCTAGGATCGATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCCAATTCACTGAGTGTACGATGTGAGTA
   AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGGTATAGATGCGCACCTCCACCA
   ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGAGGTGGT

901 TCGTCACAGTTCTGCGCCTCAAGTGCACCGGTGATGCATCTAATGC
   AGCAGTGTAAAAGACCGGGAGTTCACGATGCCACTACGTAGGATTACG

951 TGATGTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG

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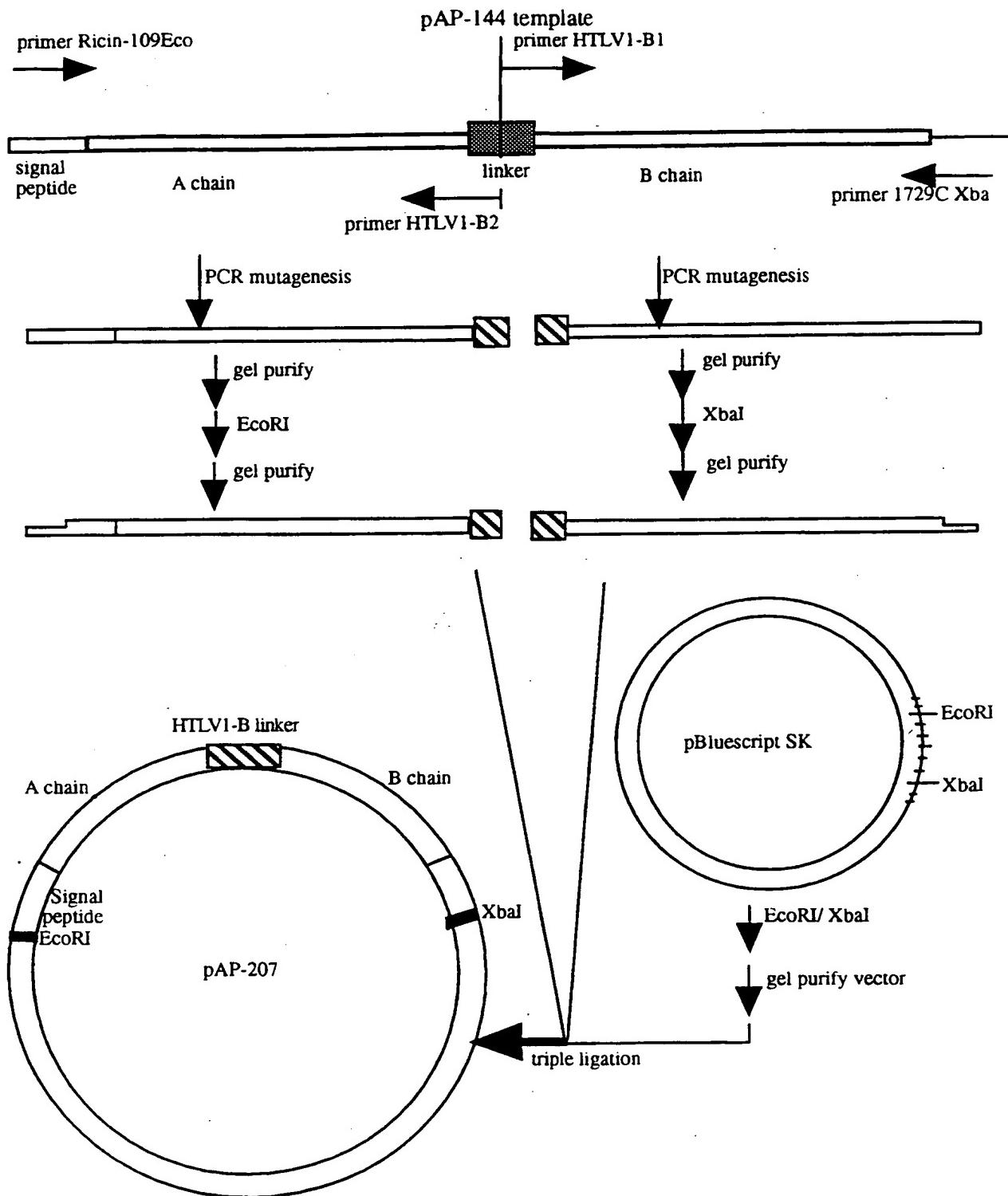
31/47

FIGURE 16D (CONT'D)

ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTTCAAGGTGTTGCCTTGCGTTAT
 1051 CAGTTGTCGGCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTACAAATTGATGAATGC
 1151 GGTACAGTCCGGAGTCTATGTGATGATCTATGATTGCAAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATAACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCCTTACCCATTACCTTGGTAGTTAG
 1251 CAGATCTAGTCTAGTTTACGAGCAGATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTGTACCATGGTGTG
 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCAACT
 AATGTCACGTTGGTTGTAACAGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACATGTTGTAACAACCCGATATACCAGACAC
 1401 CCTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
 GAACGTTGTTATCACCTGTTACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTTCAATACGTCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACGGAATGTTCACTAAGATTATATGCCCTTGTC
 1551 TGTTAAGATCCTCTTGTCGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTAACATATCACCTAACCAATCTA
 1651 GTGAGGCCATGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGAGAGGT
 1701 TGGTGAACCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
 ACCACTGGGTTGTTATACCAATGGTAATAAAACTATCTGTCAATGAG
 1751 CTCTTGCACTGTGTTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTTGTAAC TGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

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32/47

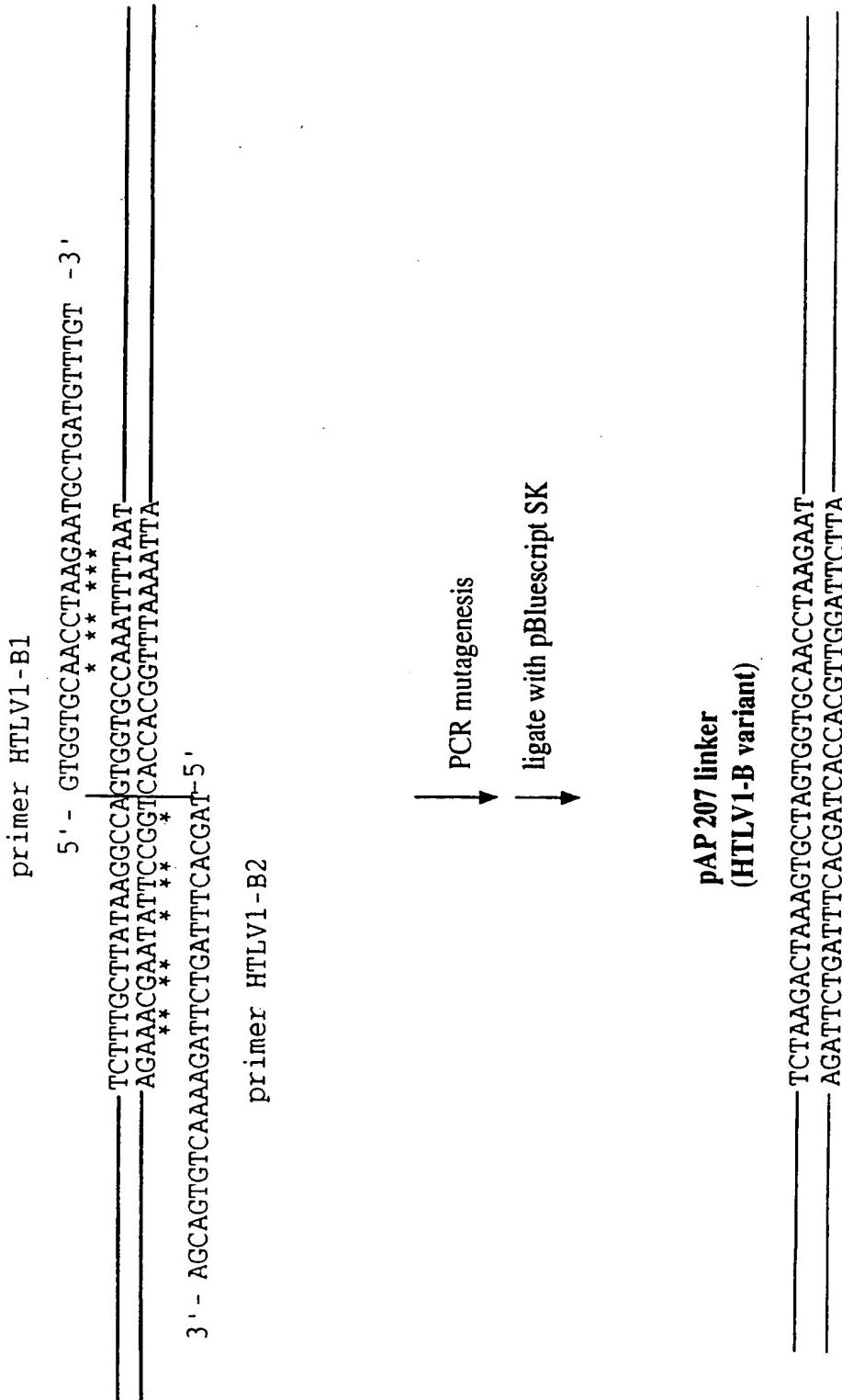
FIGURE 17A

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33 / 47

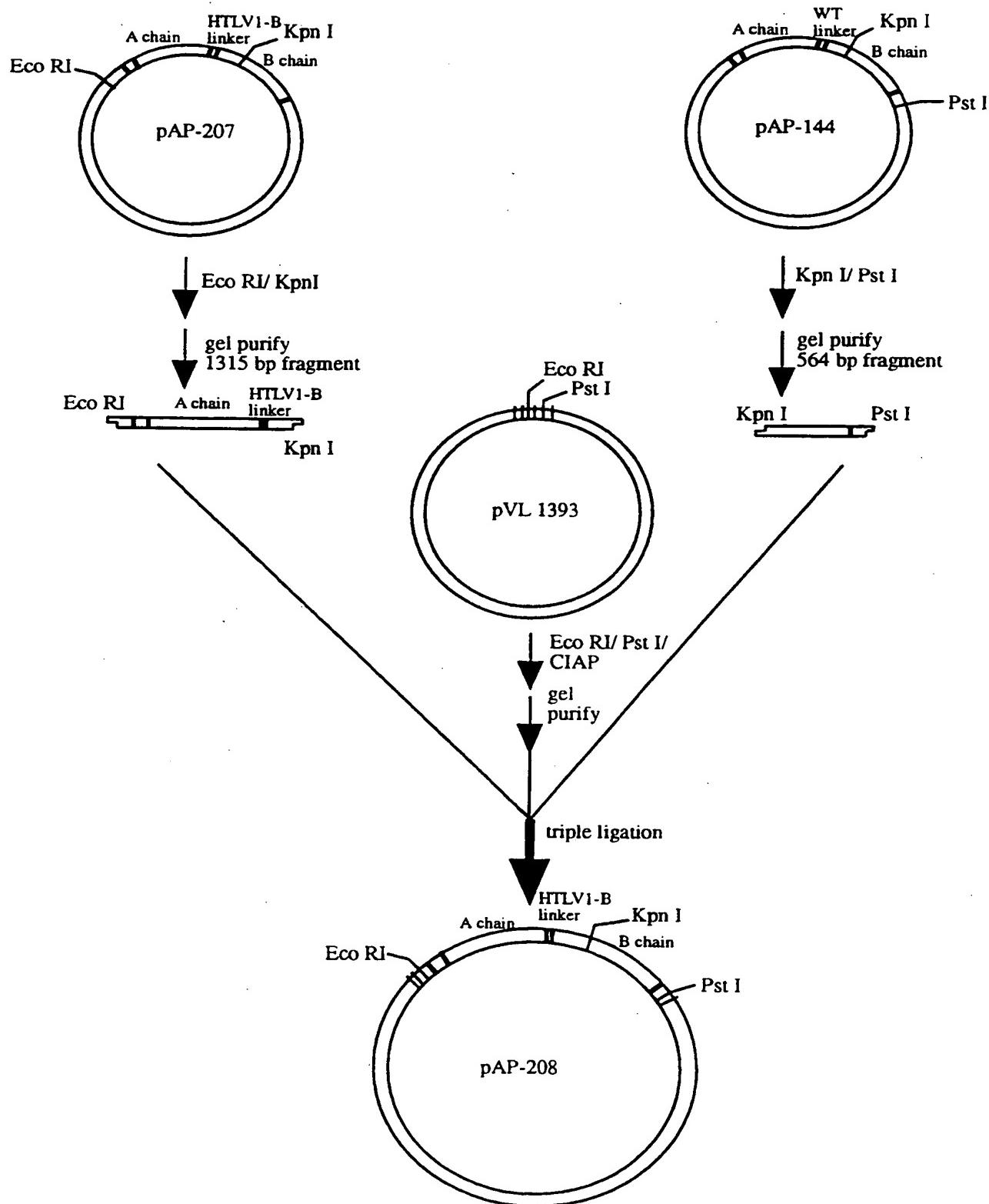
FIGURE 17B

WT preprorocin linker



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34/47

FIGURE 17C**SUBSTITUTE SHEET (RULE 26)**

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35/47

FIGURE 17D

| | | | | | | | | | | |
|---|--------------------|-----------------------|----------------------|-----------------------|------------------------|-----------------------|------------------------|----------------------|----------------------|----------------------|
| 1 | 10 GAATTCA | 10 CTTAAGTACT | 20 TTGGCCCTC | 20 TTATGATAAC | 30 ACATTACCTAC | 30 ACATACGTCA | 30 ACATTACCTAC | 40 ACAGGAAAC | 40 ACAGGAAAC | 50 ACAGGAAAC |
|---|--------------------|-----------------------|----------------------|-----------------------|------------------------|-----------------------|------------------------|----------------------|----------------------|----------------------|

1 GAATTCAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCCTTATGATAACATTACCTACATACGTCA
 51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA
 101 AGGATAACAACATATTCCCCAAACAATAACCAATTATAAACTTACCA
 TCCTATTGTTGATAAGGGTTGTTATGGGTTAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTTGC
 CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTGGTTGCCTATAACCAACGGTTATTAGTTAGTTGAACCTCTA
 TGTCTAACCAACCGGATATTGGTTGCCAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAACACTCATCTTTCACTGATGTTCAAAT
 TAGTCCTCTACGCTCTCGTAGTGGAGTAGAAAAGTAGACTACAAGTTTA
 451 CGATATACATTGCCTTGGTGGTAATTATGATAGACTTGAAACAACCTGC
 GCTATATGTAAGCGAAACCAACCATTAATACTATCTGAACCTGTTGAACCG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTAACCCCTTACCGGTGATCTCCTCC
 551 CTATCTAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAACT
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGCAATCCAATGATTCAGAACGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTACCGGTGCTTTAACCATGTTGGCCT
 701 GATCTGCAACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
 CTAGACGTGGCTAGGATCGCATTATGTAACCTTATCAACCCCTCT
 751 CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCAAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGTGTGACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGGTGGAGGTGGT
 901 TCGTCACAGTTCTAACGACTAAAGTCTAGTGGTCAACCTAACGAAATGC
 AGCACTGTCAAAAGATTCTGATTTCACGATCACCAACGTTGGATTCTACG
 951 TGATGTTGTATGGATCCTGAGCCCATAAGTGTGATCGTAGGTGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

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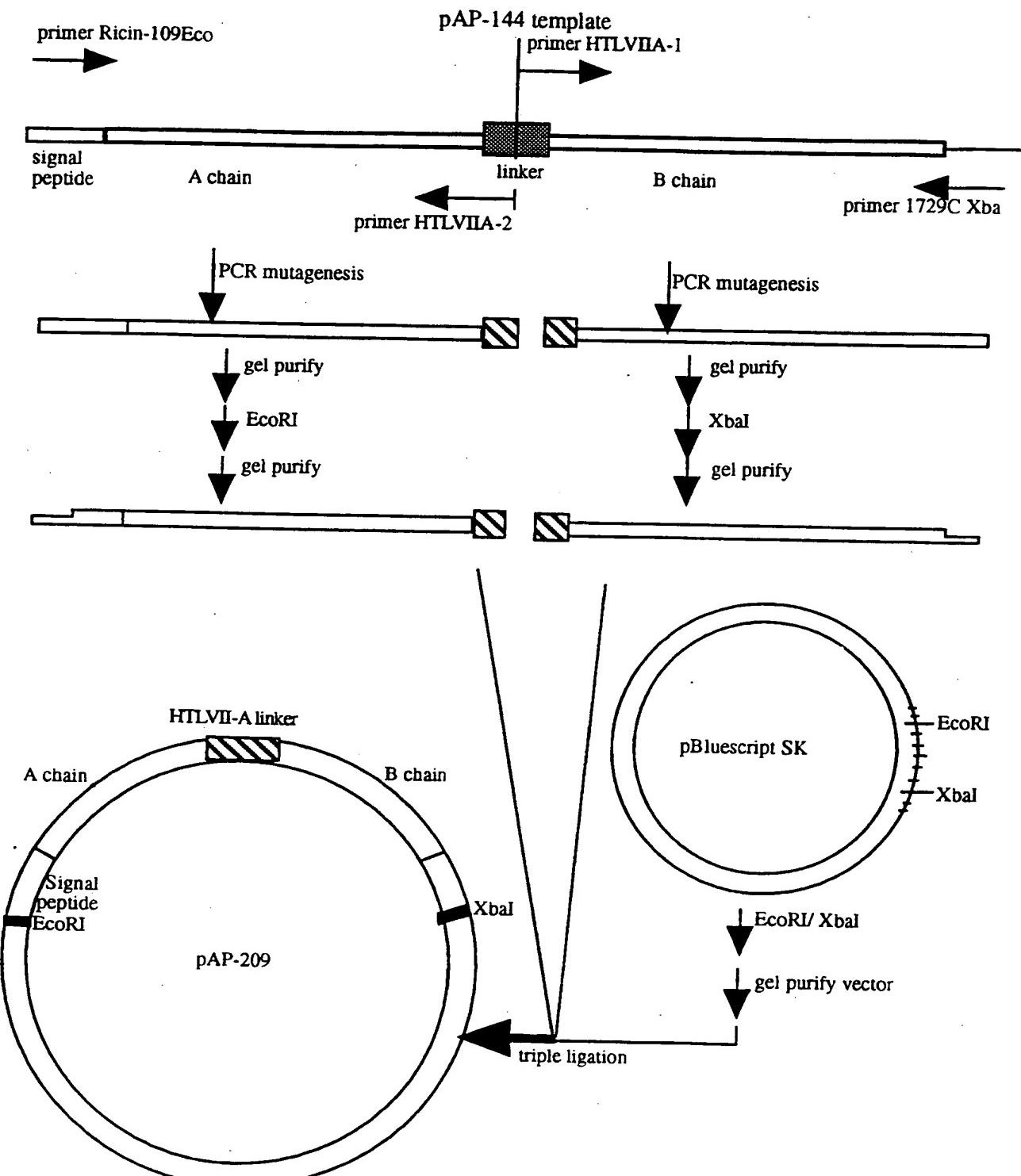
36/47

FIGURE 17D (CONT'D)

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTTCTAAGGTGTTGCCCTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTTAACTACTTACG
 CTTTCTCTGTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATAACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAACAGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATGGTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACATGTTGTAACAAACCCGATATACCAAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTCACCTATCTCCTGACATCGTCACCTT
 1451 AGGCTGAACAAACAGTGGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCACCAAGTTATGCAAGGAGTC
 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACGGAATGTTCAACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
 AGTTCTTACTACCTTGGTAAATTAAACATATCACCTAACCAATCTA
 1651 GTGAGGCATCGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCAAACCAAATATGGTTACCAATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA
 1751 CTCTTGCACTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

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37/47

FIGURE 18A

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38 / 47

FIGURE 18B

WT preprorocin linker

primer HTLV11-A1

5' - GTGGTGCACAACTAGGAATGCTGATGTTGT - 3'
 * * * * *
 TCTTGCTTATAAGGCCAGTGGTGCCTAAATTAAAT
 AGAACGAAATTCCGGTACCCACGGTTAAATTA
 * * * * *
 3' - AGCAGTGTCAAAAGATTCTGATTTCACCGAT - 5'

primer HTLV11-A2

PCR mutagenesis

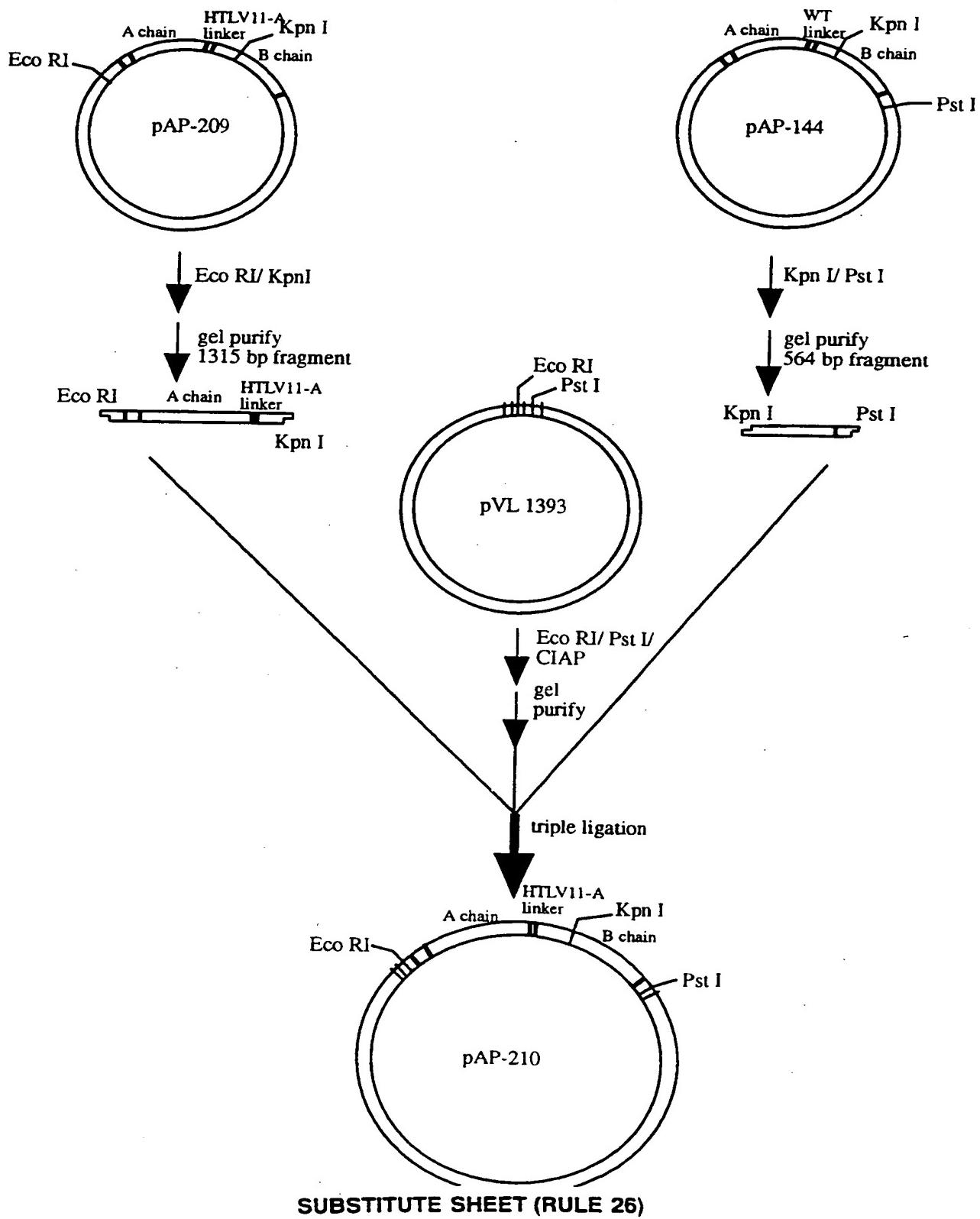
ligate with pBluescript SK

pAP209 linker
(HTLV11-A variant)

— TCTAAAGACTAAAGTGTAGTGGTAGCTAACCTAGGAAT-
— AGATTCTGATTTCACGATCACCAGTTGGATCCCTTA-

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39/47

FIGURE 18C

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40/47

FIGURE 18D

| 10 | 20 | 30 | 40 | 50 |
|---|----|----|----|----|
| 1 GAATT CATGAAACCGGGAGGAAATACTATTGTAAATATGGATGTATGCAGT | | | | |
| CTTAAGTACTTTGGCCCTCCTTATGATAACATTACACATACAGTCA | | | | |
| 51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTTCACATTAG | | | | |
| CCGTTGTAACGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA | | | | |
| 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCA | | | | |
| TCCTATTGTTGTATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT | | | | |
| 151 GCGGGTGCCACTGTGCAAAGCTACACAAACCTTATCAGAGCTGTTGCC | | | | |
| CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCACGACAAGCGCC | | | | |
| 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCA | | | | |
| AGCAAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT | | | | |
| 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA | | | | |
| TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT | | | | |
| 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCAATGCATA | | | | |
| TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT | | | | |
| 351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA | | | | |
| ACACCAGCCGATGGCACGACCTTATCGCGTATAAAGAAAGTAGGACTGT | | | | |
| 401 ATCAGGAAGATGCAAGCAATCACTCATCTTTCACTGATGTTCAAAAT | | | | |
| TAGTCCTTCTACGTCTCGTTAGTGAAGTAGAAAGTGAACACTAAGTTTA | | | | |
| 451 CGATATACATTGCCCTTGGGTAATTATGATAGACTTGAAACAACCTGC | | | | |
| GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG | | | | |
| 501 TGGTAATCTGAGAGAAAAATCGAGTTGGAAATGGTCCACTAGAGGAGG | | | | |
| ACCATTAGACTCTTTATAGCTAACCTTACCAAGGTGATCTCCTCC | | | | |
| 551 CTATCTCGCGTTATTACAGTACTGGTGGACTCAGCTTCCAAC | | | | |
| GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA | | | | |
| 601 CTGGCTCGTCTTATAATTGATCCAAATGATTTCAGAAGCAGCAAG | | | | |
| GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT | | | | |
| 651 ATTCCAAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA | | | | |
| TAAGGTATATAACTCCCTCTTACCGTGCTCTTAATCCATGTTGGCCT | | | | |
| 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA | | | | |
| CTAGACGTGGCTAGGATCGCATTATGTGAACCTTATCAACCCCTCT | | | | |
| 751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT | | | | |
| GAAAGGTGACGTTAAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA | | | | |
| 801 TCAACTGCAAAGACGTAATGGTCAAATTCACTGAGAATAGTTGGGGAGA | | | | |
| AGTTGACGTCTCGCATTACCAAGGTTAAGTCACACATGCTACACTCAT | | | | |
| 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA | | | | |
| ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGGAGGTGGT | | | | |
| 901 TCGTCACAGTTCTAAGACTAAAGCTAGTGGTCAACCTAGGAATGC | | | | |
| AGCAGTGTCAAAGATTCTGATTTCACGATCACCACGTTGGATCCTTACG | | | | |
| 951 TGATGTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG | | | | |
| ACTACAAACATACCTAGGACTCGGGTATCAGCAGCATAGCAGCTTTAC | | | | |

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41/47

FIGURE 18D (CONT'D)

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCCTTGCGTTAT

 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAACAGGGCAATCAGTTCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
 AACGTTCGTTATCACCTGTCACCTATCTCCTGACATCGTCACTTT

 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACGTCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCACCAAGTTATGCAGGAGTC

 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTAAACGGATGTCACTAAGATTATGCCCTTGTC

 1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTCTTACTACCTGGTAAAATTAAACATATCACCTAACCAACATCTA

 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

 1701 TGGTGACCCAAACCAAATATGGTTACCAATTGATAGACAGATTACT
 ACCACTGGGTTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA

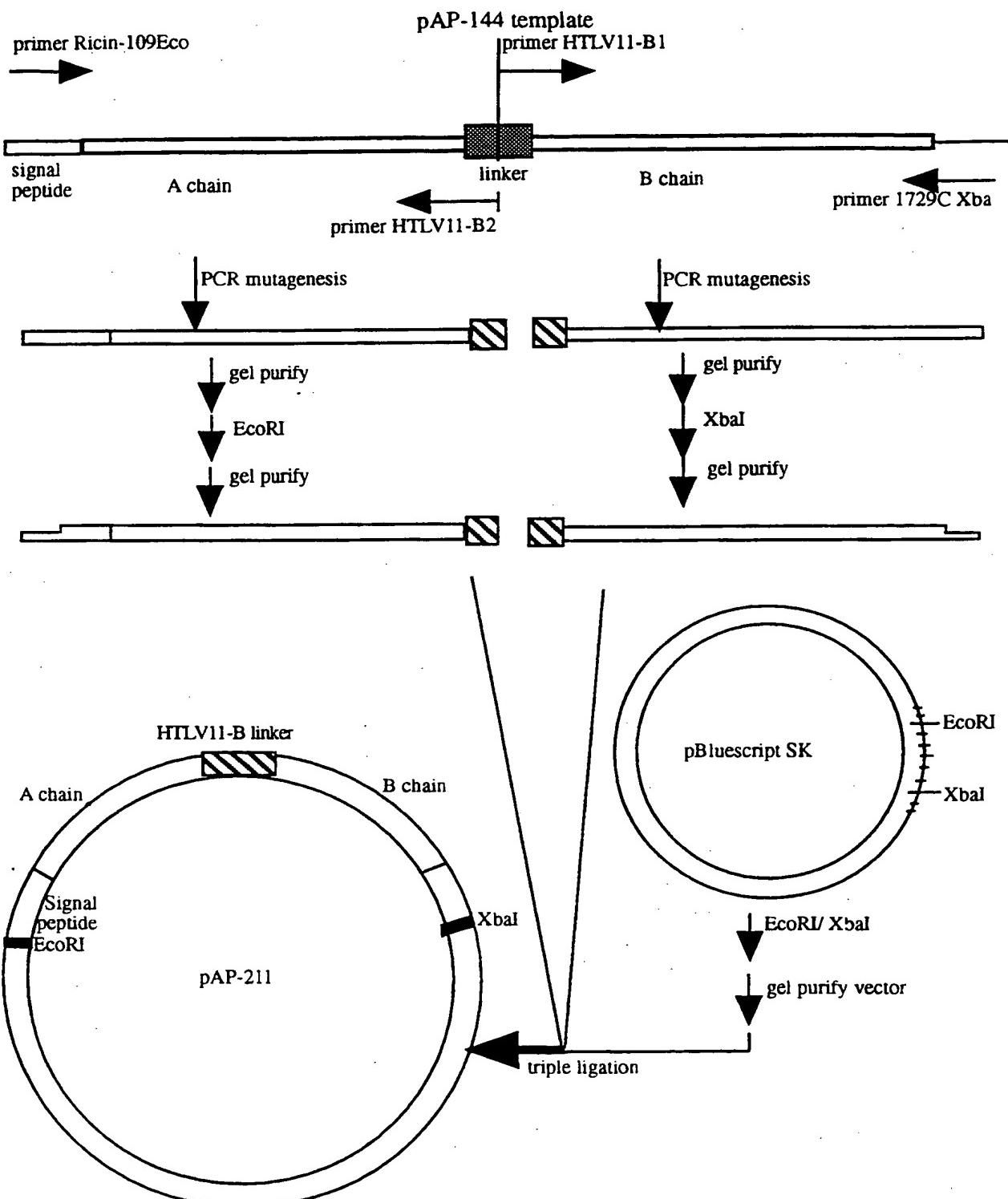
 1751 CTCTTGAGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCCTGTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

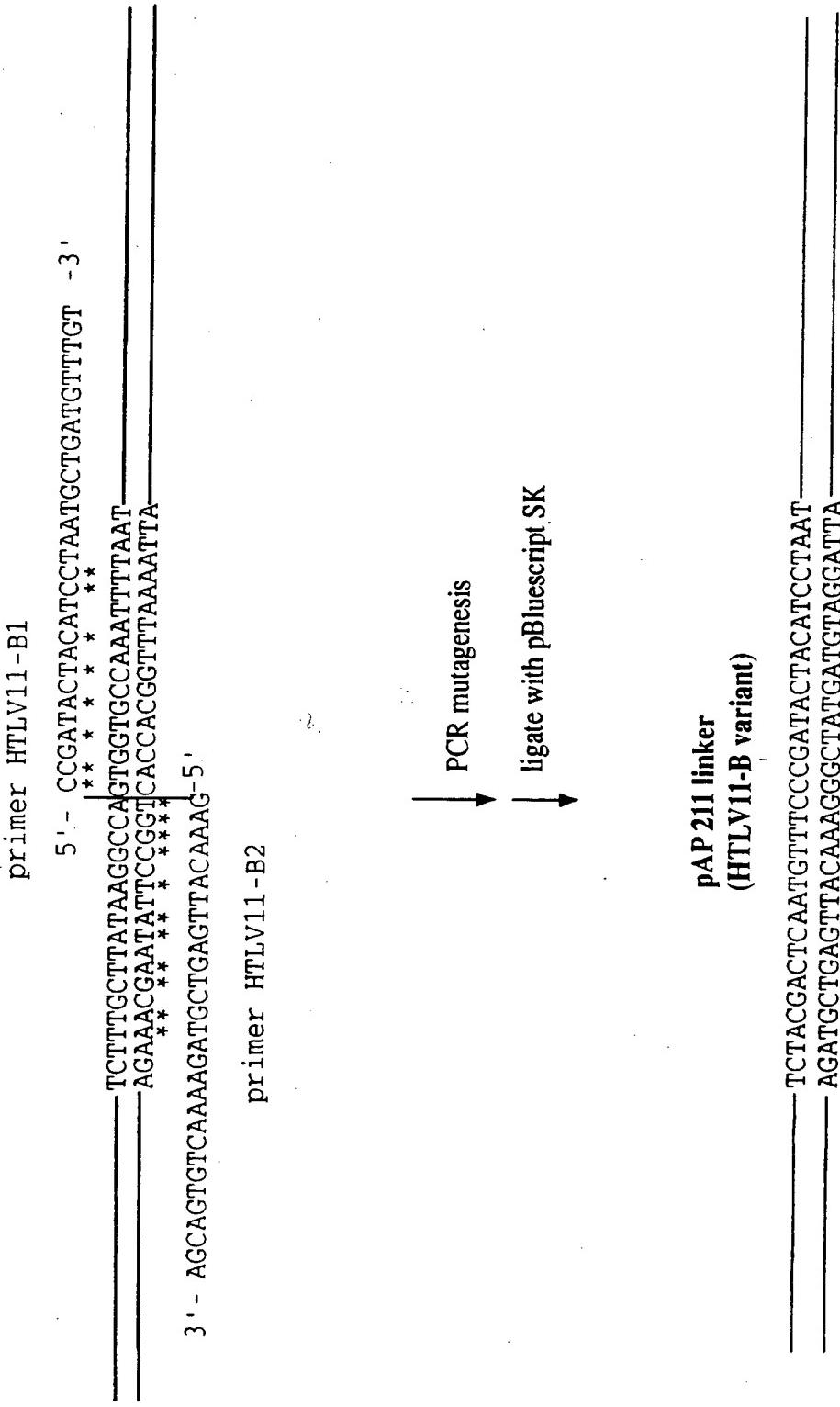
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42/47

FIGURE 19A

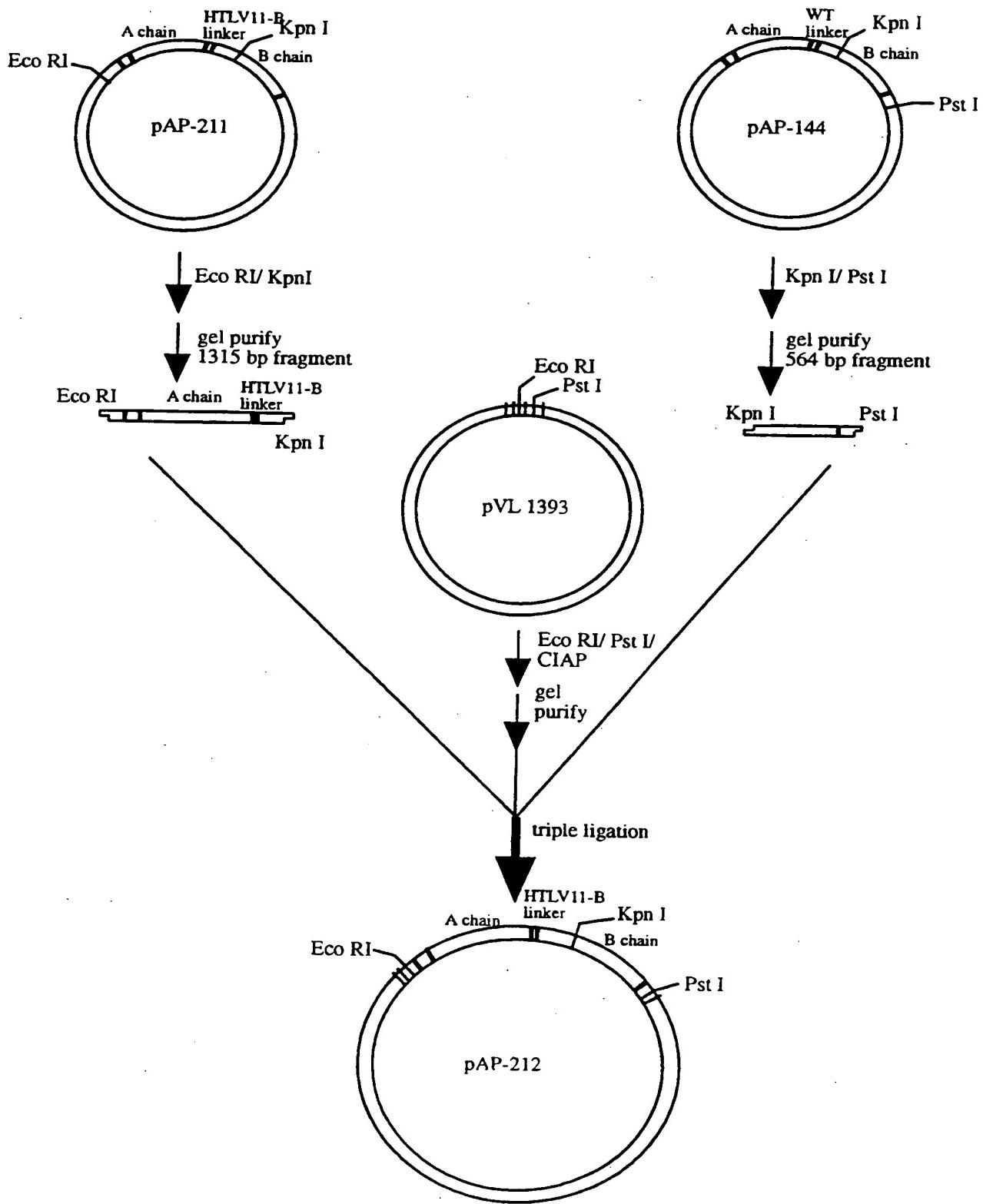
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43/47

FIGURE 19B**WT preprorocin linker**

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44/47

FIGURE 19C

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45/47

FIGURE 19D

10 20 30 40 50

1 GAATT CATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCCTTATGATAACATTACCTACATACGTCA
 51 GGCAACATGGCTTGGATCCACCTCAGGGTGGTCTTCACATTAG
 CGGTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAAACATATTCCCCAAACAATACCCAAATTATAAAACTTACCA
 TCCTATTGTTGATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG
 CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTCAACCAACGGATATTGGTGCACAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGTACCGTGTGGAAAATAGCGCATATTCTTCATCCTGACA
 ACACCAGCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAAT
 TAGTCCTTCTACGTCTCGTTAGTGAGTAGAAAAGTGAACACTACAAGTTTA
 451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAAACAACCTGC
 GCTATATGTAAGCGGAAACCACCAATTAAACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTTTATAGCTAACCTTACAGGTGATCTCCTCC
 551 CTATCTAGCGCTTATTATACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCCTTATAATTGCAATCCAAATGATTCAGAACGAGCAAG
 GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTCGTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTTTACCGTGTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTATGTAACCTTATCAACCCCTCT
 751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAAATGGTCCAAATTCACTGAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTAGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGAGGTGGT
 901 TCGTCACAGTTTCTACGACTCAATGTTCCGATACTACATCCTAATGC
 AGCAGTGTCAAAAGATGCTGAGTTACAAAGGGCTATGATGTAGGATTACG
 951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

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46/47
FIGURE 19D (CONT'D)

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
 CAGATACACAACATACTACCTTCTAAGGTGTTGCCCTTGCGTTAT

 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCCGGTACGTTACGATTATGCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCCTTATACCTTATTACCTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTACAGCAGCAGATCAGGGAACAGTGGTACCAAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTGCACCAACACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACATGTTGTAACAAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT

 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTGTTGTCACCCGAGAAATACGTCACCAAGTTATGCAGGAGTC

 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGCTCTATTAACGGAATGTTACTAAGATTATATGCCCTTGTCA

 1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACCAATCTA

 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

 1701 TGGTGAACCAAACAAATATGGTACCAATTATTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCAGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

 1801 GGACATTGTAATTGTAACTGAAAGGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

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47/47

FIGURE 20

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-205 linker: A chain- S A P Q V L P V M H P N -B chain
pAP-206
(HTLV1-A linker)

pAP-207 linker: A chain- S K T K V L V V Q P K N -B chain
pAP-208
(HTLV1-B linker)

pAP-209 linker: A chain- S I R K I L F L D G I N -B chain
pAP-210
(HTLVII-A linker)

pAP-211 linker: A chain- S T T Q C F P I L H P N -B chain
pAP-212
(HTLVII-B linker)

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INTERNATIONAL SEARCH REPORT

| |
|---|
| International Application No PCT/CA 97/00288 |
|---|

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|---|
| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/29 C12N15/62 C12N15/70 C12N15/86 A61K38/16 |
|---|

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| Y | WESTBY M ET AL: "PREPARATION AND CHARACTERIZATION OF RECOMBINANT PRORICIN CONTAINING AN ALTERNATIVE PROTEASE-SENSITIVE LINKER SEQUENCE" BIOCONJUGATE CHEMISTRY, vol. 3, no. 5, 1 January 1992, pages 375-381, XP000578216 cited in the application see the whole document --- | 1-28 |
| Y | LEPPLA S. ET AL.: "Development of anthrax-toxin based fusion proteins for targeting of HIV-1-infected cells" ZENTRALBLATT FÜR BAKTERIOLOGIE, vol. 24, 1994, pages 431-442, XP002041056 see the whole document --- | 1-28 |
| | -/- | |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

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|---|--|
| Date of the actual completion of the international search 1 October 1997 | Date of mailing of the international search report 15.10.97 |
|---|--|

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Fax: (+31-70) 340-3016

Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

Inte
onal Application No
PCT/CA 97/00288

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|--|-----------------------|
| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | PETTIT S. ET AL.: "Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the P1 amino acid" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 22, 5 August 1991, pages 14539-14547, XP002041058 cited in the application see the whole document --- | 5,8,18, 20 |
| A | EP 0 466 222 A (DOWELANCO) 15 January 1992 cited in the application see the whole document --- | 1-28 |
| A | O'HARE M. ET AL.: "Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence" FEBS LETTERS, vol. 273, no. 1,2, 29 October 1990, pages 200-204, XP002041057 cited in the application see the whole document --- | 1-28 |
| A | COOK J P ET AL: "BIOLOGICALLY ACTIVE INTERLEUKIN 2-RICIN A CHAIN FUSION PROTEINS MAY REQUIRE INTRACELLULAR PROTEOLYTIC CLEAVAGE TO EXHIBIT A CYTOTOXIC EFFECT" BIOCONJUGATE CHEMISTRY, vol. 4, no. 6, 1 November 1993, pages 440-447, XP000417282 see the whole document ---- | 1-28 |
| A | WO 89 01037 A (CETUS CORP) 9 February 1989 see the whole document ----- | 1-28 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 97/00288

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 21-24 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 97/00288

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|--|------------------|---|--|
| EP 0466222 A | 15-01-92 | US 5248606 A AU 638133 B AU 7832991 A CA 2044201 A CN 1062172 A JP 4279599 A US 5635384 A US 5646026 A | 28-09-93 17-06-93 12-12-91 12-12-91 24-06-92 05-10-92 03-06-97 08-07-97 |
| WO 8901037 A | 09-02-89 | AU 2136788 A | 01-03-89 |